Journal of DENTAL RESEARCH

FOUNDED BY WILLIAM J. GIES IN 1919

Official Publication of the
International Association for Dental Research

EDITOR FRANK J. ORLAND, University of Chicago

ADVISORY EDITORIAL BOARD

W. D. Armstrong	J. Gibilisco	A. L. Morris	J. J. Salley
G. Bevelander	S. C. Harris	T. Ockerse	R. M. Stephan
N. W. Chilton	H. A. Hunter	F. D. Ostrander	D. A. Wallace
C. O. Dummett	J. R. Jarabak	G. H. Rovelstad	A. H. Wuehrmann
A. K. Fisher	E. Johansen	G. Ryge	B. J. Zeldow

ASSOCIATE EDITORS David F. Mitchell and Richard S. Manly.

EDITORIAL ASSISTANT Kay Campbell McGrath.

PUBLICATION COMMITTEE J. A. English, S. Y. Kreshover, R. S. Manly, D. F. Mitchell, J. C. Muhler, *ex officio*, F. J. Orland, *chairman*.

VOLUME 40

1961

DENTAL
RK1
.J86
D4
COPY 2

Copyright 1961 by The International Association for Dental Research
Composed and Printed at the University of Chicago Press
Chicago, Illinois, U.S.A.

CONTENTS OF VOLUME 40

JANUARY–FEBRUARY, 1961 • NO. 1

1 Editorial Views and News

2 Announcement of 1961 General Meeting of the International Association for Dental Research

3 Factors Influencing Experimental Carcinogenesis in the Hamster Cheek Pouch—ALVIN L. MORRIS

16 Sucrose Retardation of Acid Etching in Dental Enamel: An Electron-microscopic Study —R. QUENTIN BLACKWELL, L. MCMILLAN, and L. S. FOSDICK

23 Studies on Periodontal Disease in the Mouse. II. Genetic and Maternal Effects—PAUL N. BAER, LYMAN B. CRITTENDEN, GEORGE E. JAY, JR., and JACOB E. LIEBERMAN

34 Relationship of Tooth Size to Cusp Number and Groove Conformation of Occlusal Surface Patterns of Lower Molar Teeth—ALBERT A. DAHLBERG

39 Physical Properties of the Zinc Oxide–Eugenol Impression Pastes—G. E. MYERS and F. A. PEYTON

49 Electron Microscopy of Enamel and Dentin of Teeth of the *Odontaspis* (Selachii)— WILSON DA SILVA SASSO and HELENA DE SOUZA SANTOS

58 Hereditary Generalized Microdontia—ARTHUR G. STEINBERG, JOSEPH F. WARREN, and LEONARD M. WARREN

63 Effect of Microstructure on the Physical Properties of Cobalt-Base Alloys—KAMAL ASGAR and FLOYD A. PEYTON

73 Effect of Casting Conditions on Some Mechanical Properties of Cobalt-Base Alloys— KAMAL ASGAR and FLOYD A. PEYTON

87 Effect of Dietary Casein-Sucrose Ratios on Amino Acid Composition of Dentinal Protein —W. C. HESS, A. DHARIWAL, J. F. CHAMBLISS, JR., and Z. C. ALBA

90 A Stannous Fluoride, Silex, Silicone Dental Prophylaxis Paste with Anticariogenic Potentialities—VINCENT A. SEGRETO, NORMAN O. HARRIS, and WARREN R. HESTER

97 Vascular Anastomoses in the Region of the Mandibular Symphysis—DAVID R. REDDEN, JACK G. BISHOP, JAMES L. MATTHEWS, and HOMER L. DORMAN

105 An Investigation of Enamel Thickness in Human Lower Incisor Teeth—BARRIE GILLINGS and MICHAEL BUONOCORE

119 Thickness of Enamel at the Base of Pits and Fissures in Human Molars and Bicuspids— BARRIE GILLINGS and MICHAEL BUONOCORE

134 Bacterial Invasion of Non-vital Dentin—I. M. CHIRNSIDE

141 Periodic Acid–Schiff Reactive Components of Human Gingiva—S. D. SCHULTZ-HAUDT, S. PAUS, and S. ASSEV

148 A Centrifugal Technique of Measuring Food Retention—DONALD J. BECK and BASIL G. BIBBY

161 A Modified Anthrone Colorimetric Technique for Use in Investigations Related to the Cariogenicity of Foodstuffs—DONALD J. BECK and BASIL G. BIBBY

171 Proteolytic Activities of Some Oral Spirochetes—ROBERT R. OMATA and EDWARD G. HAMPP

177 Penetration of Carcinogenic Hydrocarbons into Oral Tissues as Observed by Fluorescence Microscopy—JOHN J. SALLEY

185 Enamel Decalcification in Food-Saliva Mixtures—NARENDAR N. SONI and BASIL G. BIBBY

190 Comparative Hydrolysis of Certain Dental Local Anesthetics by Human Serum and Plasma—CHARLES E. BECKER

195 Electrophoretic Separation of Human Serum Protein That Hydrolyzes Local Anesthetics —CHARLES E. BECKER

199 Carbonyl-binding Compounds as Inhibitors of Experimental Caries in Rats—HAROLD V. JORDAN, ROBERT J. FITZGERALD, and NORMA D. BERGER

204 Studies of the Growth of the Upper Facial Skeleton Using Radioactive Calcium—ANDREW D. DIXON

RESEARCH ANNOTATIONS

217 Experimental Studies on Neural Mechanisms of Bruxism—YOJIRO KAWAMURA, SHUSAKU TSUKAMOTO, and KIYOKATSU MIYOSHI

218 Comparison of Caries Incidence in Exercised and Immobilized Rats—RALPH R. STEINMAN, MORRIS BRUSSETT, and PETER TARTARYN

219 Positive Birefringence in Non-carious Enamel—BARBARA JEFFRIES WALLACE

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

220 Sections and Officers

222 Supporting Associates

MARCH–APRIL, 1961 • NO. 2

223 Editorial Views and News

225 International Association for Dental Research—Abstracts of Papers Presented at the Seventh Annual Meeting of the Japanese Division

232 Use of Roentgenograms and Lightgrams as Supplements to Autoradiographs of Tooth Sections—ROBERT E. GOING, MAURY MASSLER, and HAROLD L. DUTE

235 Electron-microscopic Observations on Soft Carious Human Dentin—ERLING JOHANSEN and HAROLD F. PARKS

249 Effects of Heat and Sonic Oscillation on Cytochrome c—PHILIP PERSON and ALBERT FINE

258 Silicate Cements and Acid Production—RICHARD H. ROYDHOUSE

264 Effect of Tetracycline on the Transmission of Dental Caries in Rats—RACHEL H. LARSON and I. ZIPKIN

268 Transitional Ameloblastic Activity Zone in Mice Teeth—HAROLD S. FLEMING

282 Ionized Calcium in Saliva—LOIS LIGHTFOOT and T. B. COOLIDGE

287 The Effect of Longitudinal Restrictive Force on Hygroscopic Expansion—JOHN S. SHELL

294 The Effect of Certain Additive Agents on the Physical Properties of Zinc Oxide–Eugenol Mixtures—RALPH W. PHILLIPS and DWAIN R. LOVE

304 Cytochemical and Biochemical Studies of Aerobic Oxidative Metabolism of Human Gingiva—PHILIP PERSON, S. SIGMUND STAHL, and SYLVIA SCAPA

311 Ultrafilterable Calcium in Caries-susceptible and Caries-immune Subjects—LOIS LIGHTFOOT, F. C. BESIC, FRANCES HARNACH, and T. B. COOLIDGE

314 The Pathways of the Early Carious Process. I. Histologic Study—THOMAS B. COOLIDGE and BARBARA JEFFRIES WALLACE

321 The Pathways of the Early Carious Process. II. Radiologic Study—BARBARA JEFFRIES WALLACE

327 Effects of Vitamin A Deficiency on Experimental Carcinogenesis in Submandibular Glands of Hamsters—A. P. CHAUDHRY, LEON SINGER, R. J. GORLIN, and R. A. VICKERS

331 Studies in the Design and Analysis of Dental Experiments. III. Sequential Analysis (Double Dichotomy)—NEAL W. CHILTON, JOHN W. FERTIG, and AUSTIN H. KUTSCHER

341 A Study of the Exfoliative Cytology of the Hard Palate and Buccal Mucosa Following Cessation of Smoking in Previous Smokers—GEORGE JAY WRUBEL and IRWIN W. SCOPP

346 The Endogenous Respiratory Quotient of Bovine Dental Pulp—ALTON K. FISHER and CHRISTIAN SCHWABE

352 The Importance of the Genotype on Susceptibility to Dental Caries in the Rat—S. ROSEN, H. R. HUNT, and C. A. HOPPERT

355 Nitrate Reduction in Whole Saliva—PAUL W. GOAZ and HELEN A. BISWELL

366 Postnatal Dentofacial Changes Induced in Rats by Prenatal Riboflavin Deficiency—FREDERICK M. DEUSCHLE, EVA TAKACS, and JOSEF WARKANY

RESEARCH ANNOTATIONS

378 The Pathogenic Role of a Rat Oral Streptococcus in Incipient Dental Caries—RALPH R. STEINMAN and JACK D. ZWEMER

379 Retention of Carbohydrate from Sugar Solutions by Salivary Sediment—R. S. MANLY

380 Effect of Diet on Alveolar Bone Resorption—F. J. MCCLURE

381 Use of Radioactive Enamel Solubility Studies—DONALD J. BECK, BARRIE R. D. GILLINGS, PAUL W. KLEIN, and BASIL G. BIBBY

382 Identification of Decalcified Area by Autoradiography of Neutron-bombarded Tooth Sections—BARRIE R. D. GILLINGS and DONALD J. BECK

383 Investigation of the Usefulness of Neutron Activation Analysis for Studying Trace Elements in Saliva—DONALD J. BECK and BARRIE R. D. GILLINGS

384 Glycolysis Inhibition with Arylalkanols—R. S. MANLY

385 Possible Etiologic Factors in Dental Erosion—THEO ROST and ALLAN G. BRODIE

386 The Effect of Tissue Extracts on the Growth of Certain Bacteria—THOMAS A. NEVIN, PAUL N. BAER, and NORMAN LITTLETON

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

387 Sections and Officers

389 Supporting Associates

MAY–JUNE, 1961 • NO. 3

391 Editorial Views and News

392 Announcement of 1962 General Meeting of the International Association for Dental Research

393 Effect of Fluoride Ingestion on the Size of the Cusp of Carabelli during Tooth Formation—GERALD J. COX, SIDNEY B. FINN, and DAVID B. AST

396 Personality Correlates of Periodontal Disease—ERNEST G. BAKER, G. HAMILTON CROOK, and ELSBETH D. SCHWABACHER

404 Cutting Studies at Air-Turbine Speeds—J. O. SEMMELMAN, P. R. KULP, and L. KURLANSIK

411 Thermal Conductivity of Tooth Structure, Dental Cements, and Amalgam—R. G. CRAIG and F. A. PEYTON

419 A Study of Posterior Mandibular Movements from Intercuspal Occlusal Position—WILLIAM L. KYDD and ALLAN SANDER

426 Experimental Carcinogenesis in Submandibular Glands of Hamsters—A. P. CHAUDHRY, D. H. REYNOLDS, R. J. GORLIN, and R. A. VICKERS

433 Amount, pH, Buffer Effects, and Amylolytic Activity of Parotid Saliva between and Immediately Following Meals—BO KRASSE

436 Histochemistry of Oral Mucous Membrane: Total Protein, Sulfhydryls, Disulfides, Ribonucleic Acid, and Desoxyribonucleic Acid—LOUIS A. CANCELLARO, JULES KLINGSBERG, and EARL O. BUTCHER

446 Studies on the Antibacterial Action of Human Saliva. II. Observations on the Mode of Action of a Lactobacillus Bactericidin—BERNARD J. ZELDOW

454 Studies on the Amino Acid Decarboxylases of Oral Lactobacilli—WILLIAM R. BIBB and W. R. STRAUGHN

461 The Comparative Distribution of Glycogen, Succinic Dehydrogenase, and Acid Mucopolysaccharides in Different-aged Animals—JULES KLINGSBERG, LOUIS A. CANCELLARO, and EARL O. BUTCHER

470 Standardized Paper Electrophoresis of Parotid Saliva: A Study of Variables in Technique and Flow Rate as Factors in Resolution—SHELDON HOLEN, NORMAN HABER, and IRMA TUCK-WEISS

479 Whole Saliva, Salivary Sediment, and Supernatant as Fermenting Agents for Foods—DONALD J. BECK and BASIL G. BIBBY

486 Acid Production during the Fermentation of Starches by Saliva—DONALD J. BECK and BASIL G. BIBBY

492 A Comparison of Two Types of Clinical Examinations: The Effect on Observed Caries Prevalence and Increments—P. R. Weisenstein, John K. Peterson, and Paul E. Norris

497 Evaluation of Techniques for Analyzing Cavity Design for Amalgam Restoration—David B. Mahler, Louis G. Terkla, and Leonard N. Johnson

504 Further Studies on the Presence of Se^{75} in Rat Saliva and Teeth after Intracardiac Injection of Radioactive Sodium Selenite—C. K. Claycomb, F. M. Sorenson, D. C. Gatewood, E. B. Jump, and M. E. Weaver

511 The Influence of Antibiotics on the Periodontal Syndrome in the Rice Rat—James H. Shaw, Derrick Griffiths, and Aina M. Auskaps

520 Changes in the Connective Tissue of Rats Fed Toxic Diets Containing Molybdenum Salts—C. A. Ostrom, R. Van Reen, and C. W. Miller

529 Additional Studies on Fluoride Uptake by Enamel from Certain Dental Materials—Richard D. Norman, James R. Platt, Ralph W. Phillips, and Marjorie L. Swartz

538 Eruption of Irradiation-produced Rootless Teeth in Monkeys—Joseph M. Gowgiel

548 Pre-eruptive Factors of Tooth Rotation and Axial Inclination—Young H. Kim, F. R. Shiere, and H. R. Fogels

558 The Caries-Conduciveness of a High-Sucrose Diet with and without the Addition of Brewer's Yeast—John Haldi, Winfrey Wynn, and Mary L. Law

561 Dissolution Rates of Enamel and Dentin in Acid Buffers—Michael G. Buonocore

571 Phosphatase in Micro-organisms Cultured from Carious Dentin and Calculus—W. H. Bowen

578 Effect of Organic Ions on Solubility of Enamel and Dentin in Acid Buffers—Theodore A. Koulourides and Michael G. Buonocore

594 Dental Caries and Nutrition in Eskimo Scouts of the Alaska National Guard—A. L. Russell, C. Frank Consolazio, and Carl L. White

604 Periodontal Disease and Nutrition in Eskimo Scouts of the Alaska National Guard—A. L. Russell, C. Frank Consolazio, and Carl L. White

614 Relation of Protein, Carbohydrate, and Fat Intake to the Periodontal Syndrome—James H. Shaw and Derrick Griffiths

RESEARCH ANNOTATIONS

622 Natural or Derived Materials Which Replace Ascitic Fluid for the Growth of an Oral Spirochete—T. A. Nevin and H. V. Jordan

623 Toxicity Study of Stannous Fluoride in Swiss Strain Mice—V. A. Segreto, R. A. Yeary, R. Brooks, and N. O. Harris

624 Cytology and Morphogenesis of *Entamoeba Gingivalis*—Wayne W. Wantland, Edna M. Wantland, and John W. Remo

625 The Nasal Septum: An Endochondral Growth Center—Louis J. Baume

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

627 Sections and Officers

628 Supporting Associates

JULY–AUGUST, 1961 • NO. 4

629 Editorial Views and News

631 Presidential Inaugural Address: International Aspects of Dental Research—JAMES A. ENGLISH

641 International Association for Dental Research: Abstracts of Papers Presented at the Thirty-ninth General Meeting

Clinical Studies, Nos. 1-13
Pathology, Nos. 14–26
Anatomy and Histology, Nos. 27–39
Nutrition, Nos. 40–52
Dental Caries I, Nos. 53–65
Microbiology I, Nos. 66–78
Electron Microscopy, Nos. 79–91
E. H. Hatton Award Competition I, Nos. 92–101
Pharmacology, Nos. 102–14
Histochemistry, Nos. 115–27
Saliva and Salivary Glands I, Nos. 128–40
Radiobiology and Oral Biology I, Nos. 141–53
Dental Caries II, Nos. 154–66
Calcification, Nos. 167–79
Periodontology, Nos. 180–92
Fluoride Studies I, Nos. 193–204
Microbiology II, Nos. 205–17
Saliva and Salivary Glands II, Nos. 218–30
E. H. Hatton Award Competition II, Nos. 231–39
Fluoride Studies II and Enamel Solubility, Nos. 240–52
Growth and Development, Nos. 253–65
Physiology, Nos. 266–78
Surgery and Clinical Studies, Nos. 279–91
Biochemistry and Enzymology, Nos. 292–304
Calculus and Plaque Formation, Nos. 305–16
Pulp Studies and Problems Related to Stress, Nos. 317–27
Oral Biology II, Nos. 328–38
Symposium, Nos. S1–S2
Dental Materials Group, Nos. M1–M44

778 Index of Participants and Sequence Numbers for Abstracts

781 Proceedings of the Thirty-ninth General Meeting: Boston, Massachusetts, March 23–26, 1961

789 Ascorbic Acid Plasma Levels and Gingival Health in Personnel Wintering Over in Antarctica—MAX J. PERLITSH, ARNE G. NIELSEN, and WILLIAM R. STANMEYER

800 A Study of *Leptotrichia buccalis*. I. Morphology and Preliminary Observations—GEORGE J. KASAI

812 Variations in the Morphology of Mandibular First Molars of Albino Rats—LEIF B. JOHANNESSEN

819 The Effectiveness of Selection in Producing Laboratory Stocks Genetically Uniform for Resistance to Dental Caries—H. R. HUNT, C. A. HOPPERT, and S. ROSEN

827 Caries Patterns of Intact and Sialoadenectomized Albino Rats of the Emory Wistar Strain Fed a Cariogenic Diet—WINFREY WYNN, JOHN HALDI, and MARY L. LAW

833 Studies on the Intermediary Carbohydrate Metabolism in Rat Oral Mucosa—JULES M. SNITZER

838 Histological, Histochemical, and Electron-microscopic Observations on the Postnatal Development of the Major Sublingual Gland of the Rat—C. R. LEESON and W. G. BOOTH

846 Some Histopathologic Changes in the Hamster as the Result of a Continuously Acting Orthodontic Appliance—HUGH I. MYERS and WILLIAM P. WYATT

RESEARCH ANNOTATIONS

857 Studies on the Non-enzymatic Release of Nitrogenous Material from Dentin—JACOB YARDENI and MICHAEL ARTMAN

858 A Histologic Study of the Effects of Several Periodontal Dressings on Periosteal-covered and Denuded Bone—P. N. BAER and F. W. WERTHEIMER

859 Silicone Rubber as a Laboratory Duplicating Material—GEORGE W. BARNHART

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

861 Section and Officers

862 Supporting Associates

SEPTEMBER–OCTOBER, 1961 • NO. 5

863 Editorial Views and News

864 Announcement of 1962 General Meeting of the International Association for Dental Research

865 Fluorine Content of Superficial Enamel Layer and Its Correlation with the Fluorine Content of Saliva, Tooth Age, and DMFT Count—ITZHAC GEDALIA, KURT A. ROSENZWEIG, and ABRAHAM SADEH

870 Effects of Hypervitaminosis D on the Periodontium of the Hamster—H. FAHMY, W. E. ROGERS, D. F. MITCHELL, and H. E. BREWER

878 Testing of Antioxidants against Experimental Caries in Rats—H. V. JORDAN, A. E. BOWLER, and N. D. BERGER

884 The Role of Electrical Potential Differences in the Etiology of Chronic Diseases of the Oral Mucosa—JÁNOS INOVAY and JOLÁN BÁNÓCZY

891 A Method for Study of Penetration of Enamel by Acid Buffers—R. S. MANLY, W. H. EMERSON, and K. K. KAPUR

903 Studies on the Inorganic Carbon Dioxide Component of Human Enamel. II. The Effect of Acid on Enamel CO_2—MARGUERITE F. LITTLE

915 Studies on the Carbon Dioxide Component of Human Enamel. III. The Effect of Neutral and Acid Fluoride—MARGUERITE F. LITTLE and JANET ROWLEY

921 Mast-Cell Density in Diphenylhydantoin Sodium Gingival Hyperplasia—C. O. DUMMETT, T. E. BOLDEN, and J. C. ASHHURST

929 Radiation-induced Changes in the Epithelium of the Buccal Mucosa—LAWRENCE P. CHASE, PATRICK D. TOTO, and MARION F. MAGALOTTI

936 Compressive Properties of Enamel, Dental Cements, and Gold—R. G. CRAIG, F. A. PEYTON, and D. W. JOHNSON

946 Caries Resistance in Golden Hamsters—P. J. HOLLOWAY

951 Conditions Affecting Sensitivity of Techniques for Detection of Bacteremia—I. B. BENDER, SAMUEL SELTZER, GERTRUDE MELOFF, and R. S. PRESSMAN

960 Dentinal and Pulpal Response. V. Application of Pressure to Exposed Dentin—MARTIN BRÄNNSTRÖM

971 Carbonic Anhydrase in Dental Tissue and the Effects of Parathyroid Hormone and Fluoride on Its Activity—KENICHI KONDO and KANAME KURIAKI

975 The Effects of Some Chemicals on Cervix Dentis—MAMORU YAMADA and SANYA SAKADA

988 Effects of Temperature Change on Mandibular Blood Flow—E. EARL MOORE, JACK G. BISHOP, J. LESTER MATTHEWS, and HOMER L. DORMAN

993 Oral Disease in Yeshiva Students—KURT A. ROSENZWEIG and ANSELM LANGER

999 Permeability of Investment at the Casting Temperature—JOHN S. SHELL and EDWARD R. DOOTZ

1004 The Pattern of the Mineralization of Enamel—JAMES K. AVERY, ROGER L. VISSER, and DONALD E. KNAPP

1020 The Effect of the Administration of Tetracycline on the Development of Teeth—GERRIT BEVELANDER, GLORIA K. ROLLE, and SIDNEY Q. COHLAN

1025 The Effect of Cow's Milk on Dental Caries in the Rat—SAMUEL DREIZEN, JO G. DREIZEN, and ROBERT E. STONE

1029 The Dental Tissues of Wild and Laboratory-raised Hibernating and Non-hibernating 13-lined Ground Squirrels—ROBERT L. RICHARDSON, ALTON K. FISHER, and G. EDGAR FOLK, JR.

1036 Meperidine and Promethazine Hydrochloride for Handicapped Patients—MANUEL M. ALBUM

1042 Mechanics in the Production of Mandibular Fractures: A Study with the "Stresscoat" Technique. I. Symphyseal Impacts—DONALD F. HUELKE

1057 The Accuracy of Large Castings—R. H. ROYDHOUSE and E. W. SKINNER

RESEARCH ANNOTATIONS

1079 A Study of Tooth Development by Tetracycline-induced Fluorescence—PHILIP J. BOYNE and CLARENCE W. MILLER

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

1081 Sections and Officers

1082 Supporting Associates

1083 Instructions to Authors

NOVEMBER–DECEMBER, 1961 • NO. 6

1085 Editorial Views and News

1086 Announcement of 1962 General Meeting of the International Association for Dental Research

1087 Studies on Dental Plaque. I. The Effect of Different Concentrations of Glucose on the pH of Dental Plaque in Vivo—I. KLEINBERG

1112 The Principle of Bacterial Antagonism Applied as an Aid in the Reduction of Dental Caries—R. R. RUTTER, W. G. RUEFENACHT, C. R. CHAMBERLAIN, P. R. THOMASSEN, M. ROSE, and C. A. SCRIVENER

1116 Micro-indentation Hardness—G. RYGE, D. E. FOLEY, and C. W. FAIRHURST

1127 Fate of Subcutaneous Anorganic Bone Implants—P. D. TOTO and J. GIANNINI

1136 An Impact Test for Dental Porcelain—P. R. KULP, P. W. LEE, and J. E. FOX

1142 The Effects of Continued Exposure to Added Increments of Water-borne or Food-borne Fluoride upon the Rat—R. E. WUTHIER and P. G. PHILLIPS

1155 Relative Frequency of Loss of Individual Molars of Rats during a Longevity Study—R. L. SHIRLEY, H. D. WALLACE, and G. K. DAVIS

1160 Fate of Homologous and Isologous Fresh and Stored Bone Transplanted to the Anterior Eye Chamber of Inbred Mice—O. B. WISWELL and F. B. WILLARD

1170 Tubular Enamel in Some Sea Fishes in Southern Africa—T. OCKERSE

1174 Effect of Surface Alteration on the Permeability of Enamel to a Lactate Buffer—K. K. KAPUR, E. FISCHER, and R. S. MANLY

1183 A Quantitative Analysis of the Decrease in Width of the Mandibular Arch during Forced Movements of the Mandible—J. A. MCDOWELL and C. P. REGLI

1186 Influence of Occlusal Wear and Age on Formation of Dentin and Size of Pulp Chamber—G. G. PHILIPPAS

1199 Reactions of Tin (II) and Fluoride Ions with Etched Enamel—W. E. COOLEY

1211 Delayed Effects of Multiple Sublethal Doses of Total Body X-Irradiation on the Periodontium and Teeth of Mice—R. C. GREULICH and B. H. ERSHOFF

1225 Synthesis and Secretion of Amylase by Rat Parotid Gland Slices—G. W. RAPP

1231 Epithelial Activity in the Exfoliation of the Deciduous Tooth and the Emerging Permanent Tooth—H. W. JOHNSON

1242 The Relative Form of the Palate in Class I and Class II Malocclusions—A. E. STAAB

1248 The Formation of Collagen Fibrils in the Dental Pulp—J. K. AVERY and S. S. HAN

1262 Mandibular Fractures as Related to the Site of Trauma and the State of Dentition—D. F. HUELKE, A. R. BURDI, and C. E. EYMAN

1275 International Association for Dental Research: Scientific Proceedings of the British Division—*Compiled by* E. D. FARMER

RESEARCH ANNOTATIONS

1291 Succinic Dehydrogenase Activity in Odontoblasts—E. M. SWARTZ and J. A. YAEGER

1292 The Inhibition and Stimulation of Oral Lactobacilli by Caries-immune and Caries-susceptible Salivas—A. B. ADAMS and H. R. CREAMER

1293 Improved Starch-Gel Electrophoretic Resolution of Parotid-Fluid Protein—K. C. HOERMAN, S. A. MANCEWICZ, and A. F. FORZIATI

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

1295 Sections and Officers

1296 Supporting Associates

1299 Index to Volume 40

DENTAL LIBRARY
RT
.386
D4

CONTENTS

January–February, 1961. Vol. 40, No. 1

1 Editorial Views and News

2 Announcement of 1961 General Meeting of the International Association for Dental Research

3 Factors Influencing Experimental Carcinogenesis in the Hamster Cheek Pouch—ALVIN L. MORRIS

16 Sucrose Retardation of Acid Etching in Dental Enamel: An Electron-Microscopic Study —R. QUENTIN BLACKWELL, L. MCMILLAN, and L. S. FOSDICK

23 Studies on Periodontal Disease in the Mouse. II. Genetic and Maternal Effects—PAUL N. BAER, LYMAN B. CRITTENDEN, GEORGE E. JAY, JR., and JACOB E. LIEBERMAN

34 Relationship of Tooth Size to Cusp Number and Groove Conformation of Occlusal Surface Patterns of Lower Molar Teeth—ALBERT A. DAHLBERG

39 Physical Properties of the Zinc Oxide–Eugenol Impression Pastes—G. E. MYERS and F. A. PEYTON

49 Electron Microscopy of Enamel and Dentin of Teeth of the *Odontaspis* (Selachii)— WILSON DA SILVA SASSO and HELENA DE SOUZA SANTOS

58 Hereditary Generalized Microdontia—ARTHUR G. STEINBERG, JOSEPH F. WARREN, and LEONARD M. WARREN

63 Effect of Microstructure on the Physical Properties of Cobalt-Base Alloys—KAMAL ASGAR and FLOYD A. PEYTON

73 Effect of Casting Conditions on Some Mechanical Properties of Cobalt-Base Alloys— KAMAL ASGAR and FLOYD A. PEYTON

87 Effect of Dietary Casein-Sucrose Ratios on Amino Acid Composition of Dentinal Protein —W. C. HESS, A. DHARIWAL, J. F. CHAMBLISS, JR., and Z. C. ALBA

90 A Stannous Fluoride, Silex, Silicone Dental Prophylaxis Paste with Anticariogenic Potentialities—VINCENT A. SEGRETO, NORMAN O. HARRIS, and WARREN R. HESTER

97 Vascular Anastomoses in the Region of the Mandibular Symphysis—DAVID R. REDDEN, JACK G. BISHOP, JAMES L. MATTHEWS, and HOMER L. DORMAN

105 An Investigation of Enamel Thickness in Human Lower Incisor Teeth—BARRIE GILLINGS and MICHAEL BUONOCORE

119 Thickness of Enamel at the Base of Pits and Fissures in Human Molars and Biscuspids— BARRIE GILLINGS and MICHAEL BUONOCORE

134 Bacterial Invasion of Non-vital Dentin—I. M. CHIRNSIDE

141 Periodic Acid–Schiff Reactive Components of Human Gingiva—S. D. SCHULTZ-HAUDT, S. PAUS, and S. ASSEV

148 A Centrifugal Technique of Measuring Food Retention—DONALD J. BECK and BASIL G. BIBBY

161 A Modified Anthrone Colorimetric Technique for Use in Investigations Related to the Cariogenicity of Foodstuffs—DONALD J. BECK and BASIL G. BIBBY

171 Proteolytic Activities of Some Oral Spirochetes—ROBERT R. OMATA and EDWARD G. HAMPP

177 Penetration of Carcinogenic Hydrocarbons into Oral Tissues as Observed by Fluorescence Microscopy—JOHN J. SALLEY

185 Enamel Decalcification in Food-Saliva Mixtures—NARENDAR N. SONI and BASIL G. BIBBY

190 Comparative Hydrolysis of Certain Dental Local Anesthetics by Human Serum and Plasma—CHARLES E. BECKER

195 Electrophoretic Separation of Human Serum Protein That Hydrolyzes Local Anesthetics —CHARLES E. BECKER

199 Carbonyl-binding Compounds as Inhibitors of Experimental Caries in Rats—HAROLD V. JORDAN, ROBERT J. FITZGERALD, and NORMA D. BERGER

204 Studies of the Growth of the Upper Facial Skeleton Using Radioactive Calcium—ANDREW D. DIXON

RESEARCH ANNOTATIONS

217 Experimental Studies on Neural Mechanisms of Bruxism—YOJIRO KAWAMURA, SHUSAKU TSUKAMOTO, and KIYOKATSU MIYOSHI

218 Comparison of Caries Incidence in Exercised and Immobilized Rats—RALPH R. STEINMAN, MORRIS BRUSSETT, and PETER TARTARYN

219 Positive Birefringence in Non-carious Enamel—BARBARA JEFFRIES WALLACE

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

220 Sections and Officers

222 Supporting Associates

Editorial Views and News

With another annual IADR meeting (our Thirty-ninth) at the proverbial doorstep and the 1961 printed program, as well as preliminary abstracts, in every member's hand, it behooves us to look at the current effort of compiling a program not only as an entity but as an interesting arrangement of sessions, subjects, and speakers, to be compared and contrasted, say, with those of the last ten years. Perhaps we should look into the past in order better to look ahead! To recall the meetings of the last decade, the sites will be cited. The 1951 meeting occurred March 12–19 (Saturday, Sunday, and Monday morning only) at French Lick, Indiana. Thereafter we met each succeeding March at Colorado Springs, Philadelphia, French Lick, Chicago, St. Louis, Atlantic City, Detroit, San Francisco, and at Chicago last year. IADR presidents for those same years were as follows: Basil Bibby, Leonard Fosdick, Maynard Hine, Francis Arnold, George Paffenbarger, Paul Boyle, Joseph Volker, Reidar Sognnaes, Ned Williams, Hamilton Robinson, and, of course, Holmes Knighton this present year.

On the program over the last ten years certain well-defined topics were noted as indicative of some evidence of progress. There were, for example, 8 reports on fluorides ten years ago, not quite enough to comprise a complete half-session in 1951. The next year, 11 papers appeared, whereas in 1953, somehow, only 3 papers on fluorides were presented. In succeeding years the papers numbered 11, 9, 11, 14, 11, 13, 17, and at this coming 1961 meeting there are 18 scheduled. These numbers are exclusive of the 12-paper-long symposium in 1960 on the mechanism of fluoride action.

In the area of oral microbial flora, the following were the number of papers presented from 1951 through 1960, respectively: 16, 8, 9, 10, 20, 21, 19, 23, 12, 24. There are 18 for 1961. It should be understood that this superficial survey excludes "read by title" listings, since such papers were actually never presented at the meeting except on rare occasions as substitutes.

The Dental Materials group has markedly and consistently increased its total number of papers for almost every one of the last ten years. These have been arranged, for the most part, in 4 half-day sessions during this period; however, there were 3 in 1951, and in the last two years the sessions have increased to 5 and 6, with the latter number being required for the coming meeting as well. During 1951–61 there have been these numbers of Dental Material presentations inclusive of symposia: 15, 21, 26, 28, 29, 34, 36, 31, 35, 53 and 44 for the year 1961. These covered such rapidly moving topics as high-speed cutting and plastics as well as the usual solid work on metals and impression and casting materials.

It is very apparent that the electron microscope is playing an ever increasing role as a very versatile research instrument with which to elucidate oral structures. There have been symposia and extensive sessions on electron microscopy—this coming year being a good example, with a 13-paper session on Friday, March 24.

Radiobiology is rapidly growing, with a separate program section. While there were scattered papers listed in the 10 programs of the past, the intensive problems of radiation on tissues will need to be studied and reported upon at a greatly accelerated rate in the future.

It is a notable observation that the Novice Award, now named the Hatton Award, was begun during this past decade (in 1953); it now has grown to dramatic proportions and motivates excellent presentations on the part of the young researcher.

The foregoing program review has emphasized the unequivocal fact that our annual meetings are well-established entities in the area of research presentation. It is here that we display our multifarious wares by means of quantities of reports. Now for the next decade let us strive for quality, since we have just about reached the saturation point in time and space allotments for an annual meeting. What began as merely a weekend meeting now commences on Thursday and, with attendance at the contiguous A.A.D.S. meeting, occupies an entire week, but not without some enlightenment.

—F. J. O.

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

THIRTY-NINTH ANNUAL MEETING, MARCH 23, 24, 25, AND 26, 1961
HOTEL STATLER, BOSTON, MASSACHUSETTS

Persons interested in dental research and related fields are invited to attend. A modest registration fee will be charged to help defray costs of the meeting. Programs and a limited number of copies of the Preprinted Abstracts will be available at the meeting for *non-member* registrants. Members are asked to bring with them the programs and Preprinted Abstracts which will be mailed to them before the meeting.

Factors Influencing Experimental Carcinogenesis in the Hamster Cheek Pouch

*ALVIN L. MORRIS**

Department of Pathology and Division of Dental Research, University of Rochester, School of Medicine and Dentistry, Rochester, New York

Research in the field of oral cancer is the responsibility of the dental profession, whereby knowledge of the neoplastic process can be combined with an appreciation of the local environmental factors peculiar to the oral cavity. Salley[1] gave impetus to this important field of investigation when he produced the first experimental oral squamous-cell carcinoma in the cheek pouch of the hamster. The opportunity now exists to investigate experimentally the relationship of many variables in the neoplastic transformation of an oral tissue. It is important to our progress that we be able to correlate the results of successive experiments and of experiments carried out in different laboratories. This can be accomplished only if experimental variables can be controlled so that the experimental lesion can be uniformly reproduced. The need which exists for the standardization of procedures stimulated the studies which are presented here.

Salley's[2] work revealed that, of the three most commonly employed carcinogenic hydrocarbons, the application of 9,10-dimethyl-1,2-benzanthracene to the hamster cheek pouch epithelium elicited the greatest response. It was also shown that mineral oil was the vehicle of choice for that carcinogen. In order to study tissue response, it seemed necessary to standardize as much as possible factors related to tissue stimulation.[3, 4] Greenstein,[5] in discussing extrinsic carcinogenic factors, wrote:

> Thus, these and many other observations indicate that the carcinogenic potency of an agent does not reside in the nature of the agent alone but is a function of the following factors: the dosage, the nature of the vehicle, the mode and length of time of administration of the agent; the strain, the species, the sex, and the age of the test animals; the site of application, the presence of concomitant factors such as the level of essential dietary constituents and the number of animals kept in a cage, and perhaps still other as yet unknown conditions.

In an effort to determine the extent to which some of the above-mentioned factors represent variables in the experimental production of cancer in the hamster cheek pouch, three separate experiments were carried out to investigate (1) whether the age of the animal at the time of initial exposure to a carcinogen affects the response of the cheek pouch mucosa (age experiment); (2) the response of the cheek pouch to different concentrations of a carcinogen (concentration experiment); and (3) whether the frequency of application of a carcinogen affects the response of the cheek pouch mucosa (frequency experiment).

This work was carried out during the author's tenure as a United States Public Health Service Postdoctorate Research Fellow of the National Institute of Dental Research.

Received for publication July 28, 1958; revised by author September 13, 1960.

* Present address: School of Dentistry, University of Pennsylvania, Philadelphia 4, Pennsylvania.

EXPERIMENTAL PROCEDURES

Age experiment.—Forty-five 3-week-old hamsters of both sexes were divided into three littermated groups. All these animals had been born on the same day. An additional group of fifteen was composed of approximately 18-month-old hamsters. All animals eventually received a 0.5 per cent mineral oil solution of 9,10-dimethyl-1,2-benzanthracene painted on the medial wall of both pouches three times per week. The painting was begun on the first group when they were 3 weeks of age (age at weaning); the second group when 6 weeks old; the third group when 9 weeks old. The 18-month-old animals of the fourth group were painted simultaneously with the first group. The animals were carefully examined at each painting and the date of development of the initial gross lesion in each animal was recorded after the manner previously described. As the lesions progressed in size, the largest tumor in each animal was biopsied. Painting of a given animal continued until a biopsy specimen from that animal was confirmed microscopically to be squamous-cell carcinoma. The date of the biopsy was recorded in the records as the date of malignancy. At this time, application of the carcinogen was stopped, but the animal was continued on the experiment without painting until death, the date of which was also recorded.

Concentration experiment.—Sixty 5-week-old hamsters of both sexes were divided into four littermated groups. Each group received one of the following concentrations (in mineral oil) of 9,10-dimethyl-1,2-benzanthracene: 1.5 per cent, 0.5 per cent, 0.1 per cent, and 0.05 per cent. The carcinogen was applied three times per week to the medial wall of both cheek pouches. After 15 weeks of the experiment, only three animals in the 0.1 per cent group had developed lesions, and the animals of the 0.05 per cent group were entirely free of any gross changes. It was decided at that time to discontinue painting the 0.05 per cent group. Since the two groups of animals receiving the weaker concentration showed no signs that exposure to the carcinogen was going to be responsible for their death, all remaining animals were sacrificed after 20 weeks of the experiment.

Frequency experiment.—Thirty hamsters of both sexes were divided into two littermated groups of fifteen each. A 0.5 per cent mineral oil solution of 9,10-dimethyl-1,2-benzanthracene was painted three times per week on both cheek pouches of the animals of one group. The animals of the other group were similarly treated twice weekly. The methods of painting and recording of data were identical with those described for the preceding experiments. The emphasis in this experiment was on the latent period required for tumor development. Biopsies were not performed on these animals, and no microscopic study of the tumors was carried out. The experiment was terminated after all animals exhibited tumors and an estimation had been made of the relative growth rate of tumors in the two experimental groups.

The animals used in the first two experiments were Syrian hamsters (*Cricetus auratus*) representing the eighth generation of a strain of animals developed in the Rochester Inheritance Study.[6] The hamsters for the last experiment were obtained from the Albino Farms of Redbank, New Jersey. Approximately equal numbers of both sexes were used. All animals were separated by sex and kept in groups of three or four in wire-mesh cages over wood shavings. Tap water and Purina Fox Chow pellets were provided ad libitum. To this diet fresh carrots and lettuce were supplemented

weekly. Each animal was weighed once each week throughout the course of an experiment.

Detailed examination and biopsy of the hamster pouch was facilitated by the use of Johansen's immobilizer.[7] A more rapid method for exposing the pouch for application of the carcinogen was devised which utilized the retractor shown in Figure 1.

The carcinogenic hydrocarbon 9,10-domethyl-1,2-benzanthracene was obtained from the Distillation Products Division of the Eastman Kodak Company, Rochester, New

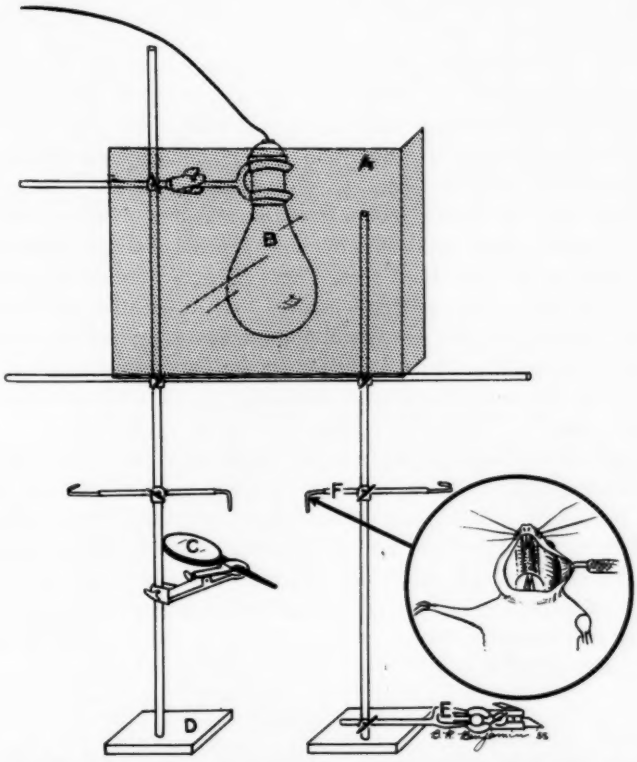


FIG. 1.—Diagram of hamster cheek pouch retractor. *A*, light shield; *B*, electric light bulb; *C*, magnifying glass; *D*, ring stand; *E*, immobilizer; *F*, retractor for lateral pouch wall. The hamster is held behind the neck and brought into contact with the retractor so that the arm (*arrow*) is inserted into the opening of the pouch. By moving the hamster horizontally, the lateral wall is retracted, exposing the interior of the pouch. (See insert.)

York. It was dissolved in U.S.P. mineral oil and kept at all times in tightly stoppered actinic glass bottles. A No. 4 camel's-hair brush was used in a circular motion to paint the carcinogen on the anterior medial wall of the hamster cheek pouch. It was desired to deliver uniform amounts of carcinogen to each animal and to use a dosage which was large enough to produce maximum carcinogenic response without using great excesses, which might increase toxicity. Two painting techniques were investigated, namely, a "dripping-brush" method and a "wiped-brush" method in which the brush was wiped once against the side of the container before passing it to the hamster

pouch. When watch glasses were weighed on an analytical balance before and after painting with each method, it was revealed that reasonable uniformity was afforded by both methods. Average amounts of solution delivered to the watch glasses were 47.7 and 31.3 mg. by the dripping- and wiped-brush methods, respectively. During an early experiment in which sixty hamsters received the carcinogen by the different methods in opposite pouches, no differences could be determined in the responses of the two pouches. The wiped-brush method was thereafter used exclusively, since it afforded less likelihood of extra-oral contamination with the carcinogen, was fast, and apparently supplied to the pouch as much as, or more than, the minimum effective dose as expressed in tumor production.

The latent period for tumor production was measured as the time required, after the initial application of the carcinogen, for appearance of the first grossly observable tumor. This period, important in the evaluation of data, depended for its accuracy on the ability to recognize the very early lesion as soon as it became manifest. The initial gross alteration of the hamster cheek pouch mucosa, indicating that a tumor would subsequently occupy a given site, was observed to take one of three forms. It was first seen as a tiny, pedunculated, reddish papilloma; a small, distinct, rounded raised pink area; or an irregular whitish raised area. These changes were designated 1, 2, and 3, respectively. The type of change first observed and its location were recorded in an animal's individual record. The area of alteration was examined at subsequent paintings, and any changes were recorded. When progressive growth into a frank tumor was confirmed, the date of the first observed change was entered into the records as the date of initial lesion.

For histologic examination, tumors were excisionally biopsied from the everted pouch in animals anesthetized by intraperitoneal injection of sodium pento-barbital (Veterinary Nembutal). After fixation in Bouin's solution, hematoxylin and eosin-stained sections were prepared in the usual manner. The histologic appearance of a normal hamster cheek pouch can be seen in Figure 2. Two early tumors exhibiting considerable histopathologic variation are shown in Figure 3. Examples of experimentally induced squamous-cell carcinomas arising in the hamster cheek pouch can be seen in Figures 4 and 5.

RESULTS

The data for the age experiment are summarized in Table 1, which reveals that whether the hamster is 3, 6, or 9 weeks of age at initial exposure to the carcinogen, it has little effect on the length of time required for initial lesion formation. A significantly greater length of time, however, is required for lesions to develop in the cheek pouch of animals which are 18 months of age when first exposed.

Determination of time required for the tumors to become malignant depended on an estimation of when to biopsy, based largely on the size of the tumors. From the data obtained it is possible to say that most of the animals had malignant tumors after approximately 35 painting (12 weeks), but this cannot be interpreted to mean that many of the biopsied tumors were not malignant much sooner.

The animals in the concentration experiment receiving the 1.5 per cent solution exhibited an extreme reaction to the carcinogen after the second application. The mild erythema and necrosis of the cheek pouch mucosa, commonly seen during the first

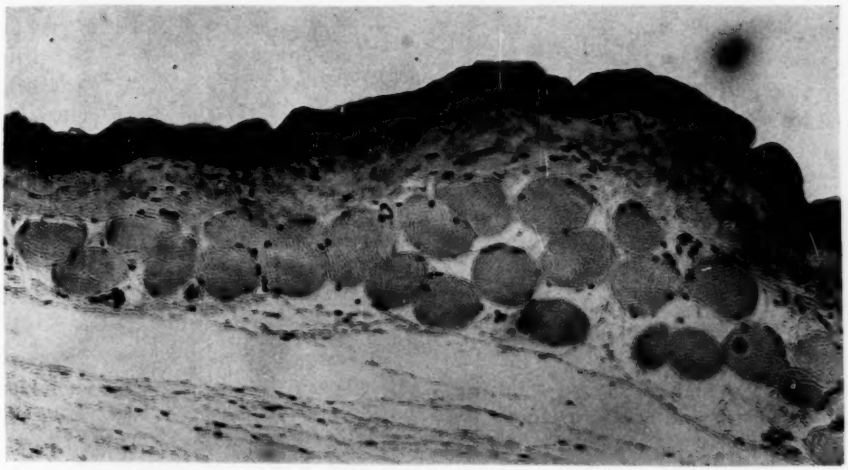


FIG. 2.—Histology of normal hamster cheek pouch. Hematoxylin and eosin. Medium power

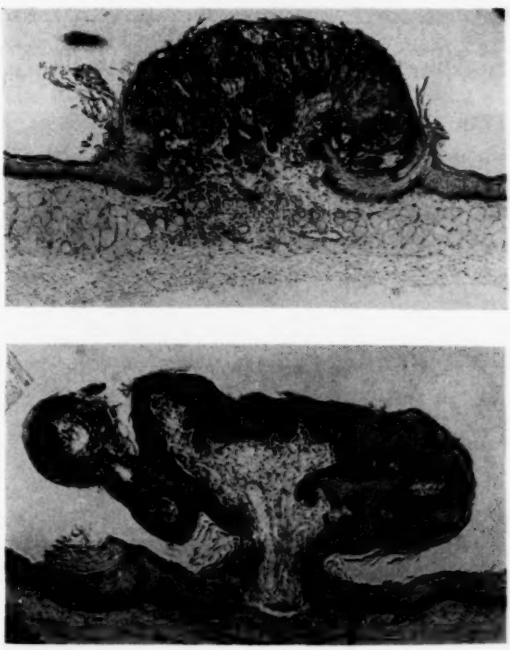


FIG. 3.—Early tumors arising in the cheek pouch of the hamster in response to repeated applications of a chemical carcinogen. Note the histopathologic variation in the two lesions. Hematoxylin and eosin. Low power.

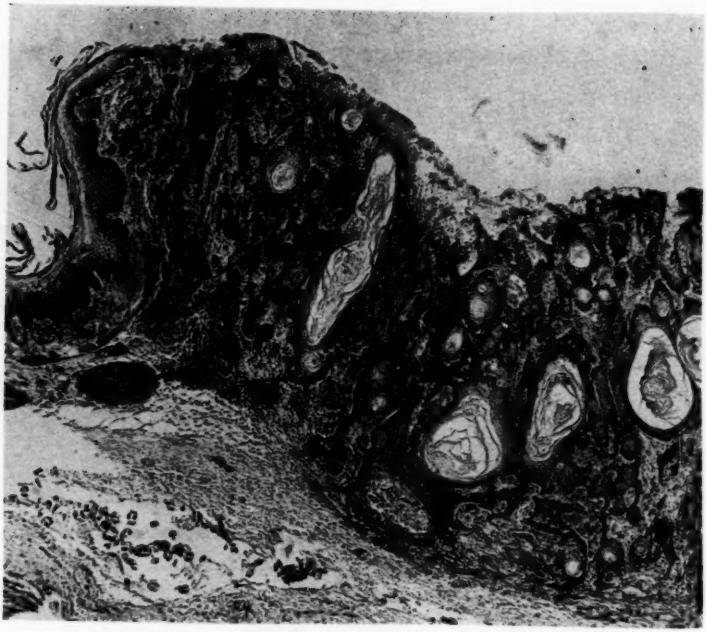


FIG. 4.—Squamous-cell carcinoma arising from the cheek pouch of the hamster. Hematoxylin and eosin. Low power.

FIG. 5.—Section taken from within a papillomatous squamous-cell carcinoma arising in the hamster cheek pouch. Hematoxylin and eosin. Low power.

week of painting in animals receiving the 0.5 per cent concentration of the carcinogen, were very severe in the 1.5 per cent group. In some of these animals the pouches never healed, and eight animals of this group died before development of initial lesions. In contrast to this observation, the animals in the 0.1 per cent group exhibited only very mild erythema of the cheek pouch mucosa. The animals receiving 0.05 per cent were without observable changes. The animals in these last two groups appeared to be in conspicuously better condition throughout the course of the experiment.

Table 2 summarizes the data of this experiment. It reveals that those hamsters which survived the early applications of the 1.5 per cent carcinogen required practically the same length of time to develop initial tumors as did the animals of the 0.5 per cent group. The hamsters in the 0.1 per cent group all developed lesions by the end of the experiment, but the latent period was markedly increased. When these animals were sacrificed after 20 weeks, it was found that the tumors in nine of the group were malignant. It was noted that the animals of the 0.05 per cent group, which had no gross evidence of tumors when the painting was stopped after 15 weeks, apparently went on to develop lesions. When these animals were sacrificed 5 weeks later, six were found to have tumors, two of which were malignant.

During the early weeks of the experiment on frequency of application of the car-

TABLE 1

NUMBER OF PAINTINGS OF 0.5 PER CENT DMBA REQUIRED TO PRODUCE INITIAL AND MALIGNANT LESIONS IN HAMSTERS GROUPED ACCORDING TO AGE AT INITIAL PAINTING

Animal Group	Initial			Malignant	
	No. of Animals	Mean No. of Paintings	*t*-Value*	No. of Animals	Mean No. of Paintings
3 week	15	16.3±2.1	.....	15	34.8
6 week	15	15.8±3.0	0.55	13	36.1
9 week	15	16.9±4.3	0.48	11	36.3
1½ year	11	20.8±3.9	3.77	9	40.6

* Compared with 3-week group.

TABLE 2

NUMBER OF PAINTINGS REQUIRED TO PRODUCE INITIAL AND MALIGNANT LESIONS IN 5-WEEK-OLD HAMSTERS GROUPED ACCORDING TO CONCENTRATION OF DMBA

Animal Group	Initial			Malignant	
	No. of Hamsters	Mean No. of Paintings	*t*-Value*	No. of Hamsters	Mean No. of Paintings
1.5 per cent DMBA	7	14.8±3.8	1.37	5	27.0
0.5 per cent DMBA	15	16.2±1.9	.....	14	37.6
0.1 per cent DMBA	14	51.6±8.4	15.9	9	59.0

* Compared with 0.5 per cent group.

cinogen it was noted that the inflammatory response of the animals receiving the carcinogen only two times per week was conspicuously milder and somewhat later in developing than the inflammation seen in the other group. Eight and one-third weeks after the initial painting, tumors were present in all animals in the group painted three times per week. (Mean number of paintings required for initial lesions was 22.) Only four animals of the group painted twice weekly had developed tumors at this time. Twelve weeks after initial painting, one animal in the three-times-per-week group exhibited tumors less than 3 mm. in diameter, ten had 3–10-mm. lesions, and three had lesions larger than 10 mm. At the same time, in the group painted twice weekly, one animal was without a tumor, three had questionable initial lesions, seven exhibited tumors smaller than 3 mm., and two had lesions larger than 3 mm. With continued painting, all animals eventually developed tumors. It was thus evident that a shorter latent period was required for tumor development in the animals exposed to a carcinogen three times per week than in those receiving the carcinogen twice weekly.

It was felt that some insight into the effect of the carcinogen on the growth and development of the hamster could be gained by observation of changes in body weight throughout the course of the experiments. That the carcinogen itself had a detrimental effect, apparently exclusive of its carcinogenic action, is supported by the following observations: (1) the older the animals were upon first receiving the carcinogen, the greater was the weight which they ultimately attained; (2) an inverse relationship was observed between the weight ultimately attained and the concentration of the carcinogen received; (3) animals receiving the carcinogen only two times per week ultimately attained greater weights than those receiving the carcinogen three times per week.

Comparison of the total days of survival of the animals revealed that age at initial exposure had little effect on the number of days an animal survived. All animals followed a similar terminal course. The size and apparently the number of the tumors in the pouches continued to increase until there was gross evidence of swelling externally. In some cases erosion of tumors through the lateral wall of the pouch occurred. The animals became increasingly emaciated until the time of death. Although a consideration of the metastatic spread of these tumors was not included in this study, it has been observed that metastasis does occur, and one can readily demonstrate the presence of tumor in the submandibular lymph nodes.

During the course of the experiments described, a total of 150 hamsters was subjected to experimental carcinogenesis. The investigator, animal facilities, experimental procedures, and recording methods were the same in all experiments. Therefore, it seemed permissible to correlate all the results in an attempt to determine the extent to which sex, heredity, and caging conditions represented variables in this experimental production of oral cancer in hamsters.

In the experiments, sixty hamsters were studied which varied in age from 3 to 9 weeks on initial exposure to a 0.5 per cent concentration of the carcinogen. Thirty-two of these animals were males; twenty-eight were females. Since age variation between 3 and 9 weeks was shown to have no effect on carcinogenesis, the variable of sex alone could be studied in this group. No difference in the response of the two sexes to carcinogenic stimulation of the cheek pouch was observed. One sex might still be more suitable for this type of experimentation if it was found to be more

resistant to the toxic effects of the carcinogen, irrespective of tumor production. No difference could be observed, however, in the survival of the sixty hamsters in question, grouped according to sex. The females were observed to attain heavier weights than the males; however, this is a routine finding in normal hamsters.

The data were reviewed on the thirty animals which received weaker concentrations of the carcinogen and on the fifteen hamsters which received the carcinogen less frequently. Here again it was not possible to demonstrate a sex difference.

The majority of hamsters used in the experiments represented the eighth generation of animals developed in the Johansen and Hodge Rochester Inheritance Study.[6] The possibility exists that a difference in cancer susceptibility has resulted from inbreeding of these animals. Such a difference was apparent when the response of these hamsters to carcinogenic stimuli was compared with that of a group of hamsters obtained from a commercial breeding farm. In this latter group the average latent period for tumor development was slightly longer. When the response of animals from different litters within either strain was studied, however, no variations could be demonstrated. It is concluded that hereditary susceptibility will not represent an experimental variable when doing cancer research on hamsters, as long as all animals on a given experiment are derived from the same strain.

In setting up experiments with hamsters, it is convenient to keep either three or four animals in each cage. It seemed desirable to rule out the possibility that conditions of crowding within a cage or relative availability of food and water could in some way affect animals being used for experimental cancer research. Individual records kept on animals used in the experiments presented here made it possible to compare the tumor production and weights of thirty hamsters, which difference could be demonstrated between the two groups.

DISCUSSION

It is of interest and importance to those working in the field of experimental oral cancer to be aware of the differences and similarities between those factors which influence the induction of neoplasia of oral tissues and those which influence neoplasia of non-oral tissues. Because of the limited amount of work to date in experimental oral cancer per se, it is necessary to relate the results of the work reported here to the findings of those working in experimental cancer in general.

There is a lack of agreement in the literature in regard to the relationship of age of animal to susceptibility or resistance to the development of experimental cancer. Some workers have concluded that age is of no importance.[8, 9] In only one of a series of experiments by Dunning and co-workers[10] did age of animals present a variable in tumor production. The increased resistance of younger animals which they observed was attributed to the fact that it required more time to break down or wear out some defense mechanism in younger cells.

Frequent claims have been made for an increased susceptibility of younger animals to tumor development. Lemon and Smakula[11] observed that sarcomas transplanted to the cheek pouch of 2–3-week-old hamsters grew at a faster rate than if the hamsters were 6–8 weeks of age at transplantation. Cowdry,[12] Cowdry, Carruthers, and Suntzeff,[13] and Cowdry and Suntzeff[14] produced epidermal cancer in mice by applica-

tion of methylcholanthrene and observed young animals to be more susceptible. They suggested a difference in the sebaceous glands or in the chemical constituents of the skin as a possible explanation for their findings. Blum and co-workers[15] induced tumors in mice by ultraviolet radiation. The induction time for old mice was longer than for younger animals receiving the same penetrability of younger skin. Strong, Smith, and Gardner[16] observed that experimental sarcomas as well as carcinomas could be more easily produced in young animals. Shimkin[17] has expressed the opinion that the greater susceptibility of young animals can be explained merely on the basis that they receive a larger carcinogenic dose per gram of body weight.

The results of the experiment reported here reveal that younger hamsters are more susceptible to experimental oral cancer. The cheek pouch does not contain sebaceous glands or other accessory structures, and so variations in these components cannot explain the age difference noted. It is doubtful that Shimkin's theory of increased dosage per gram of body weight can be applied, inasmuch as the animals in the 9-week-old group had attained the greater part of their growth before exposure to the carcinogen. It seems logical, however, that, in a tissue undergoing constructive metabolism, biochemical changes and abnormal cells would be produced earlier in response to carcinogenic stimulation.

In spite of the fact that the animals ultimately died at approximately the same age, a consideration of the weight curves suggests that the carcinogen was more toxic to the 3-week-old hamsters than to the older animals. This observation, coupled with the fact that the 3-week-old weanlings were too small to handle conveniently, makes it seem unwise to use animals of this age in future experiments. Approximately 5 weeks appears to be the age of choice from the standpoint of both ease of manipulation and tumor production.

Most reports in the literature which have dealt with variations in concentration of carcinogen have been concerned with subcutaneous injection of the carcinogen, a procedure which has doubtful application to the present experiment. Berenblum[18] and Poel,[19] however, applied various concentrations of carcinogens to the skin of several species of laboratory animals with results similar to those presented here.

The early death of eight animals in the 1.5 per cent concentration group, even before the development of initial lesions, was felt to be due to the extremely severe inflammation seen in these animals. The direct relationship observed between degree of inflammation and concentration of the carcinogen, coupled with the inverse relationship between ultimate weight attained and carcinogen concentration, was taken as evidence of the toxic nature of the carcinogen. A concentration of 0.5 per cent of the carcinogen appears to be the one of choice for the rapid development of malignant tumors, from the standpoint of both a short latent period and maximum survival of the animals.

Many systemic and local factors are believed to have an effect on the metabolism of the oral tissues. It is interesting to speculate as to how these effects may be related to cancer of the mouth. It appears that it would be difficult to demonstrate an effect of subtle changes upon the carcinogenic process by using an experimental technique which elicits a tissue response of 100 per cent tumors in 12 weeks. It seems desirable, therefore, to have a weaker concentration which can be used to produce a submaximal response that might lend itself to alterations in tumor production as a result of subtle

alterations in the host tissues. The 0.1 per cent concentration apparently qualified in this regard. The 0.05 per cent concentration did ultimately develop tumors.

It is recognized that one cannot divorce concentration from dosage. Dosage, on the other hand, is closely related to frequency of application.[20] Blum and co-workers,[15] using ultraviolet light on the skin of mice, found that the induction time was shorter when the dosage was given in more frequent intervals. Saffiotti and Shubik[21] observed that small repeated doses of carcinogen to the skin of Swiss mice were more effective than large single applications, irrespective of total quantity. The work of Cramer and Stowell[20] and Salaman and Roe[22] indicated that skin cancer can be induced in mice by smaller doses if the applications are made at longer intervals.

Evaluation of the data presented here reveals that a significantly extended latent period is required for tumor development in hamsters receiving the carcinogen only two times per week. This finding is consistent with that of Berenblum,[18] who compared the results of one and two applications per week on the skin of mice. Thus, from the standpoint of rapid development of malignancies, it is preferable to paint the animals three times per week. From the standpoint of total dosage required to produce tumors, it can be calculated (from the average mg. of carcinogen delivered per painting) that it required a smaller total dose to produce tumors in all animals when the carcinogen was applied at longer intervals. This observation is similar to those made by other workers.[20, 22]

There is a lack of agreement in the literature in regard to the effect of sex on cancer susceptibility. Dunning,[10] Branch,[23] Boyland and Warren,[24] and Kreshover[25] have reported that tissue response to chemical carcinogens is not influenced by sex. Leiter and Shear[26] and Andervont[27] found in different strains of mice that the males were more responsive. However, the former authors observed the difference only when marginal doses of the carcinogen were applied. Reinhard and Candee[28] and Kreyberg,[29] on the other hand, observed earlier tumor formation in female mice following the application of tar to the skin.

Various explanations for a sex difference have been proposed. Bullough[30] pointed out that the skin of female mice experiences a twofold increase in thickness of the epidermis and frequency of mitoses in the pro-estrus period. Wicks[31] has observed that males of several strains of mice ordinarily exhibit proteinuria, whereas females excrete much less and frequently none at all. This finding suggests that a difference may exist in the elimination of carcinogens and other materials from the body during carcinogenesis. Cowdry[12] proposes that perhaps sex difference is accomplished by a difference in the disposal within the body of the carcinogen and/or its products. Using the hamster cheek pouch under the conditions of the experiments discussed here, there was no difference in the response of the males and females to carcinogenic stimuli.

Although some workers have doubted that malignant change is influenced by the genetic constitution of the host,[10] the majority of evidence supports those who recognize strain variations in response to chemical carcinogens.[13] Numerous pure strains of mice have been developed in which spontaneous tumors are common, suggesting a hereditary influence on cancer susceptibility.[10, 12] Some of these inbred strains of mice have also displayed a greater tendency to develop tumors in response to locally applied chemical carcinogens.[23, 24, 28, 29] From the results obtained in the experiments

reported here, it was apparent that, while hereditary susceptibility may vary between strains of hamsters, it does not represent an experimental variable when all animals on a given experiment are derived from the same strain.

SUMMARY

1. Standardized experimental techniques for chemical carcinogenesis in the hamster cheek pouch are presented.

2. Under the conditions of the experiments described here, the tissues of the cheek pouch of old hamsters are more resistant to carcinogenic stimuli than those of young hamsters. No difference in rate of carcinogenic response is seen between the ages of 3 and 9 weeks. Five weeks appears to be the ideal age for hamsters used for experimental oral carcinogenesis from the standpoint of ease of manipulation and tumor production.

3. Five-tenths per cent concentration of 9,10-dimethyl-1,2-benzanthracene is the optimal concentration for the rapid production of malignant tumors in the hamster cheek pouch. This concentration produces maximum tumor response with minimum latent period, with no loss of animals due to toxicity; 0.1 per cent and 0.05 per cent concentrations will produce graded submaximal responses in tumor production.

4. A shorter latent period is required for tumor development in animals exposed to a carcinogen three times per week than in those receiving the carcinogen twice weekly. A smaller total dose is required to produce tumors in all animals when the carcinogen is applied twice each week than when given three times weekly.

5. The response of the hamsters to the repeated application of the carcinogenic hydrocarbon 9,10-dimethyl-1,2-benzanthracene is not related to the sex of the animals under the conditions of the experiments presented here.

6. Conditions of caging (three or four hamsters per cage) had no apparent effect on the experimental results obtained in the experiments reviewed here.

REFERENCES

1. Salley, J. J. Experimental Carcinogenesis in the Cheek Pouch of the Syrian Hamster, *J. D. Res.*, **33**:253, 1954.
2. ———. The Chemical Induction of Carcinoma in Oral Epithelium of the Hamster and the Rat, Ph.D. thesis, University of Rochester, New York, 1954.
3. Morris, A. L. Experimental Premalignant and Early Malignant Oral Epithelium in the Syrian Hamster. In *Proceedings of the Twenty-fifth Year Celebration of University of Rochester Dental Research Fellowship Program*, p. 81. Univ. Rochester Publication. 1957.
4. ———. Studies on the Production and Histochemistry of Experimental Oral Cancer in the Hamster, Ph.D. thesis, University of Rochester, Rochester, New York, 1957.
5. Greenstein, J. P. *Biochemistry of Cancer*, p. 43. 2d ed. New York: Academic Press, 1954.
6. Johansen, Erling, and Hodge, H. C. *Hereditary Factors in Hamster Caries: Fourth Report.* International Association of Dental Research, Thirty-fourth General Meeting, Preprinted Abstracts, No. 46, 1956.
7. Johansen, Erling. A New Technique for Oral Examination of Rodents, *J. D. Res.*, **31**:361, 1952.
8. Brunschwig, A., and Tschetter, D. Age Factor and Latent Period in Production of Sarcoma by Methylcholanthrene in Rats, *Proc. Soc. Exptl. Biol. Med.*, **36**:439, 1937.
9. Woglom, W. H. Experimental Tar Cancer, *Arch. Path.*, **2**:709, 1926.
10. Dunning, W. F., Curtis, M. R., and Bullock, F. D. The Respective Roles of Heredity and Somatic Mutation in the Origin of Malignancy, *Am. J. Cancer*, **28**:681, 1936.
11. Lemon, H. M., and Smakula, E. Factors Affecting Hamster Sarcoma Growth in the Cheek Pouch, *Cancer Research*, **15**:273, 1955.

12. COWDRY, E. V. *Epidermal Carcinogenesis*, p. 57. In J. P. GREENSTEIN and ALEXANDER HADDOW (eds.), *Advances in Cancer Research*, Vol. **1.** New York: Academic Press, 1953.
13. COWDRY, E. V., CARRUTHERS, D. C., and SUNTZEFF, V. Influence of Age on the Copper and Zinc Content in the Epidermis of Mice Undergoing Carcinogenesis with Methylcholanthrene and a Note on the Role of Calcium, *J. Natl. Cancer Inst.*, **8**:209, 1948.
14. COWDRY, E. V., and SUNTZEFF, V. Influence of Age on Epidermal Carcinogenesis Induced by Methylcholanthrene in Mice, *Yale J. Biol. and Med.*, **17**:47, 1944.
15. BLUM, H. F., GRADY, H. G., and KIRBY-SMITH, J. S. Relationships between Dosage and Rate of Tumor Induction by Ultraviolet Radiation, *J. Natl. Cancer Inst.*, **3**:91, 1942.
16. STRONG, L. C., SMITH, G. M., and GARDNER, W. V. Induction of Tumors by 3:4:5:6-Dibenzcarbazole in Male Mice of the CBA Strain, Which Develops Spontaneous Hepatoma, *Yale J. Biol. and Med.*, **10**:335, 1938.
17. SHIMKIN, M. B., Factors Influencing Carcinogenesis with Methylcholanthrene. I. The Effect of Age, *Public Health Repts.*, **54**:1388, 1939.
18. BERENBLUM, I. The Carcinogenic Action of 9,10-Dimethyl-1,2-Benzanthracene on the Skin and Subcutaneous Tissues of the Mouse, Rabbit, Rat, and Guinea Pig, *J. Natl. Cancer Inst.*, **10**:167, 1949.
19. POEL, W. E., and KAMMER, A. G. Preliminary Studies in a Quantitative Approach to Skin Carcinogenesis, *J. Natl. Cancer Inst.*, **16**:989, 1956.
20. CRAMER, W., and STOWELL, R. E. On the Quantitative Evaluation of Experimental Skin Carcinogenesis by Methylcholanthrene, *Cancer Research*, **3**:668, 1943.
21. SAFFIOTTI, UMBERTO, and SHUBIK, PHILIPPE. The Effects of Low Concentrations of Carcinogen in Epidermal Carcinogenesis: A Comparison with Promoting Agents, *J. Natl. Cancer Inst.*, **16**:961, 1956.
22. SALAMAN, M. H., and ROE, F. J. C. The Development of Malignant Tumors of Mouse Skin after "Initiating" and "Promoting" Stimuli. IV. Comparison of the Effects of Single and Divided Initiating Doses of 9,10-Dimethyl-1,2-Benzanthracene (DMBA), *Brit. J. Cancer*, **10**: 79, 1956.
23. BRANCH, C. F. Dibenzanthracene Tumors in Controlled Strains of Mice, *Am. J. Cancer*, **26**:110, 1936.
24. BOYLAND, E., and WARREN, F. L. The Induction of Tumors by Methylcholanthrene in Two Strains of Mice, *J. Pathol. Bacteriol.*, **45**:171, 1937.
25. KRESHOVER, S. J. Further Observations on the Effect of Tobacco on Epithelial Tissues of Vitamin-deficient Mice, *J. D. Res.*, **34**:798, 1955.
26. LEITER, JOSEPH, and SHEAR, M. J. Quantitative Experiments on the Production of Subcutaneous Tumors in Strain A Mice with Marginal Doses of 3,4-Benzpyrene, *J. Natl. Cancer Inst.*, **3**:455, 1943.
27. ANDERVONT, H. B. Susceptibility of Mice to Spontaneous, Induced, and Transplantable Tumors, *Public Health Repts.*, **53**:1647, 1938.
28. REINHARD, M. C., and CANDEE, C. F. Influence of Sex and Heredity on the Development of Tar Tumors, *Am. J. Cancer*, **16**:640, 1932.
29. KREYBERG, LEIV. On the Susceptibility to Cancer Development in the Skin and in the Mammary Gland in Two Lines of Inbred Mice, *Am. J. Cancer*, **24**:554, 1935.
30. BULLOUGH, W. S. Epidermal Mitotic Activity in the Adult Female Mouse, *J. Endocrinol.*, **6**: 340, 1950.
31. WICKS, L. F. Sex and Proteinuria of Mice, *Proc. Soc. Exptl. Biol. Med.*, **48**:395, 1941.

Sucrose Retardation of Acid Etching in Dental Enamel: An Electron-Microscopic Study

R. QUENTIN BLACKWELL, L. McMILLAN, and L. S. FOSDICK
Chemistry Department, Northwestern University Dental School, Chicago, Illinois

In a previous communication from this laboratory,[1] data were presented to show that the solubility of powdered human dental enamel in acid solutions was reduced in the presence of sucrose. The present communication deals with the logical extension of that work from powdered enamel to whole enamel surfaces. It will be shown that sucrose diminishes the rate of etching of tooth surfaces by lactic acid solutions. This effect, demonstrated in the present work by means of the electron microscope, is in agreement with the chemical studies reported earlier.[1]

It is well to emphasize that the following results are not presented in an effort to minimize the deleterious effects of sugar as an agent in caries development. Instead, the results are presented for the information they provide. They serve to demonstrate once again the amazing complexity of factors involved in the process of caries formation.

EXPERIMENTAL MATERIALS AND METHODS

The human teeth used for the present study were central incisors and first molars obtained from the Great Lakes Dental Research Facility of the U.S. Navy. The labial surface of the incisors and the buccal surface of the molars were used for the experimental surfaces.

The plane areas required for electron microscopy were prepared on the tooth surfaces by polishing and buffing according to a standardized procedure used in this laboratory.[2] Because it is a common procedure, it will be described only briefly. The tooth was imbedded in a cold-setting acrylic resin and polished with successively finer grades of levigated alumina followed by buffing with Sturge's heavy calcium carbonate. The polishing and buffing were carried out on a Buehler style R-1138 polishing wheel. The calcium carbonate buffing produces a reasonably standard type of plane surface in which the ends of the enamel rods are visible. Following the buffing operation the plane enamel surface was rinsed well with distilled water, wiped with lens paper, and soaked briefly in 95 per cent ethyl alcohol. The surface now was ready for examination by replication with polyvinyl alcohol (PVA) film. This film was used for the initial replicas, which were examined by means of the light microscope to establish the suitability of the surface. The PVA film also served to strip the surface of any lightly bound solid debris. In some cases the surfaces required additional buffing or were found to be unsuitable and discarded. The suitable surfaces were ready for the etching studies. In certain cases the tooth surface, after the initial use, was ground down, a new surface prepared, and the etching experiment repeated.

Received for publication November 20, 1958; revised by authors July 21, 1960.

The solutions used for the main phases of this study, all at pH 4.5, were as follows: (1) 0.1 M lactic acid buffer; (2) 0.1 M lactic acid buffer–1 M sucrose solution; (3) 0.1 M lactic acid buffer–0.5 M sucrose solution; and (4) 0.1 M lactic acid buffer–0.1 M sucrose solution. Each of the above solutions were 0.1 molar with respect to lactate. To prepare them, the required amount of freshly boiled 85–90 per cent lactic acid solution was added to the volumetric flask along with some water; the required amount of sugar was added, followed by more water to bring the solution nearly to the desired volume. Next, sufficient NaOH was added to bring the pH up to 4.5, after which the final small amount of water was added to fill the volumetric flask to the proper level.

After the above solutions were tested for their action on the enamel surfaces and the results were considered, other solutions were tested to determine the degree of specificity of the sucrose action. These solutions included (1) 0.1 M lactic acid buffer–1 M sodium chloride solution, pH 4.5; (2) 0.1 M lactic acid buffer–1 M glucose solution, pH 4.5; (3) 1 M sucrose solution, pH 7; (4) 1 M sucrose, pH adjusted to 11 with sodium hydroxide.

Over a period of several years, in pursuit of other problems, numerous combinations of pH, nature of the acid etching solution, and time intervals have been tried in this laboratory for their effectiveness in etching enamel surfaces. A considerable degree of variation in rate of etching has been found to exist among teeth. That is, several teeth subjected to the same etching conditions will show differences in their degree of etching. However, a generally useful procedure which produces reasonably marked etching of prepared enamel surfaces consists of the application of 0.1 M lactate buffer, pH 4.5 for 15 minutes. All the etching studies reported here, therefore, were done with solutions left on the prepared enamel surfaces for 15 minutes. Because of the above-mentioned natural variations in etching rates of teeth, it was considered desirable to use each tooth surface as its own control for comparison of treatments. This was done with variations in other work as well as in the present problem. In the present case the technique was to keep half of the prepared planar tooth surface covered with pressure-sensitive electrician's tape, while the other half was subjected to the 15-minute treatment with the desired solution. After water rinsing and careful blotting, the treated half was covered with tape, and the other half uncovered and treated with a second solution. In this manner, pairs of solutions were compared on individual tooth surfaces for their effect. In all cases in the present report the first half of the surface to be treated was subjected to the pure buffer solution while the adjoining half of the surface was covered with tape. After that initial treatment, the tape was removed from the untreated half-surface, while tape was applied to the previously treated surface. Next, the second half-surface was treated with the buffer-sugar or buffer-salt solution. In this manner both surfaces were covered with electrician's tape during one part of the process. However, the pure buffer-treated surface was covered after buffer treatment, whereas the buffer-sugar–treated surface was covered with tape prior to treatment. A commonly used technique to remove loose surface debris from an etched surface prior to replication is the application of a PVA film or cellulose tape to the surface. By stripping the surface, the operator can obtain in subsequent replicas a reproduction of the remaining tightly bound surface structure. In an unetched standard tooth surface prepared as described above and used in the present study, such stripping technique produces no noticeable difference in the surface, as shown by replica-

tion before and after such stripping. On the other hand, such a standard surface after a 15-minute treatment with 0.1 M lactate buffer, pH 4.5, is deeply etched. Such a surface appears quite rough upon replication either with or without stripping.

By the order in which the surfaces were treated in the present study it is apparent that the pure buffer-treated surface was stripped after treatment while the buffer-sugar–treated surface was not. Furthermore, it was conceivable that the prepared tooth surface would react differently to a solution after having been covered previously with the tape. For example, a water-repellent film might be left adhering to the surface and tend to protect the surface from etching by the solution. Therefore, any differences seen between degree of etching of the two surfaces might result from the biased treatment. Preliminary tests showed, however, that such was not the case. The effect was independent of the order. Accordingly, the standard procedure as described was adopted. In the initial experiments the 0.1 M lactate buffer, pH 4.5, was compared with the pH 4.5 lactate-sucrose solutions. In subsequent experiments the other solutions were compared with the pH 4.5 0.1 M lactate-buffer solution and with each other.

Finally, the treated surfaces were examined by electron microscopy of surface replicas. Polyvinyl alcohol (PVA) "negative" replicas, made by direct application to the tooth surface, were treated with Formvar to form the "positive" replica, after which the PVA "negative" was dissolved off the positive by water flotation. The resulting free Formvar replica was shadowed with chromium and examined in the RCA electron microscope. The degree of etching of the surface was determined simply by inspection and comparison of the two portions of each tooth surface. No attempt was made to quantitate the amount of etching. The results obtained are given below.

RESULTS

Approximately twenty tooth surfaces were studied, some of which were used two or three times (by rebuffing as described above). It was found invariably that the degree of etching caused by the pH 4.5 lactate buffer was reduced in the presence of sucrose. The portion of the enamel surface subjected to the lactate-buffer solution always showed a greater degree of etching than the adjoining portion treated with the sucrose–lactate-buffer solution. As mentioned earlier, some variation is generally found among individual teeth in the degree of etching produced by a given acid treatment. As anticipated, therefore, in the present experiments some tooth surfaces were more susceptible to etching than others. Those teeth showing somewhat less etching accordingly showed less difference between the lactate surface and the lactate-sucrose surface. That is, for the full effectiveness of the sucrose retarding action to be demonstrated, it was necessary for the tooth to be etched appreciably in the 15-minute period. In most cases the lactate-sucrose–treated surfaces showed minimal amounts of etching, while the corresponding lactate-treated surfaces showed quite appreciable etching. Although no actual quantitative means of assessing the etching was used, the 0.5 M sucrose appeared to be as effective as 1.0 M sucrose in depressing the etching, while the 0.1 M sucrose appeared to be slightly less effective.

An illustration of the typical difference between lactate-buffer and lactate-buffer–sucrose solution is provided in Figure 1. In this illustration the boundary region where the two separately treated areas of the tooth surface meet is shown. Electron-micro-

FIG. 1.—Tooth 4. Standard surface, produced as described in text. Boundary region. Surface in lower-left-hand corner treated for 15 minutes with 0.1 M lactate buffer–1 M sucrose, pH 4.5. Surface on other side treated for 15 minutes with 0.1 M lactate buffer, pH 4.5. (Approx. mag. ×1330.)

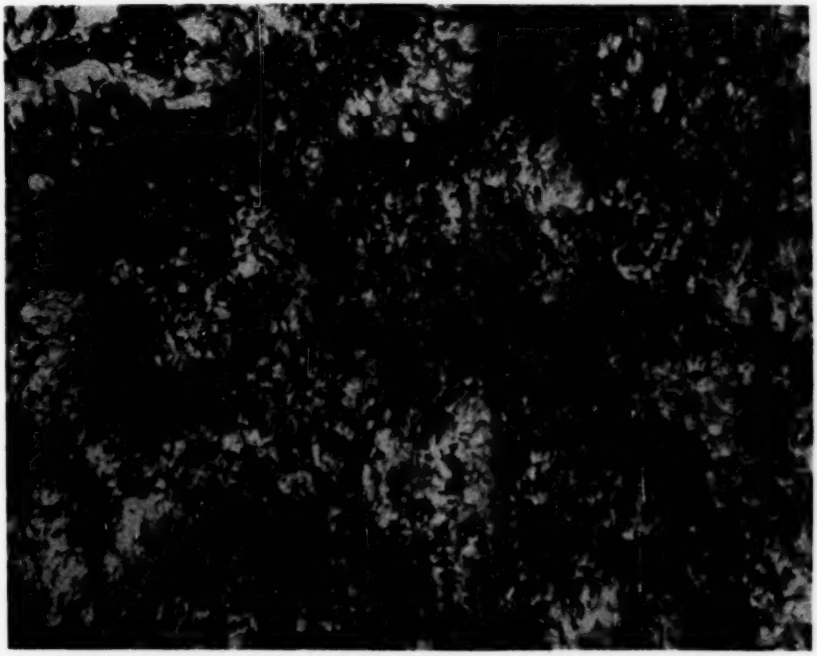


FIG. 2.—Tooth 4. Standard surface, produced as described in text. Another view of the same tooth surface shown in Figures 1 and 3. In this picture the entire area in view was treated for 15 minutes with 0.1 M lactate buffer, pH 4.5. (Approx. mag. ×3380.)

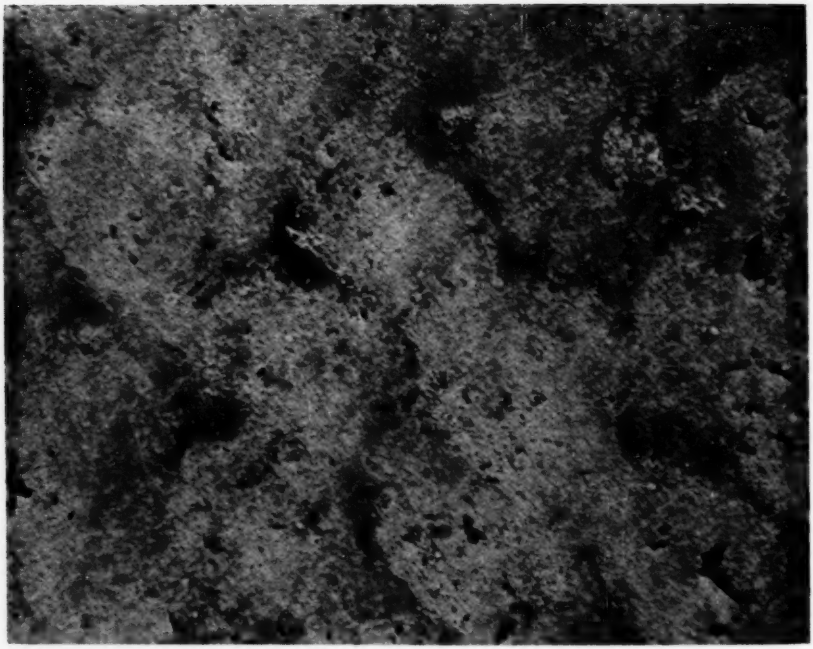


FIG. 3.—Tooth 4. Standard surface, produced as described in text. Another view of the same tooth surface shown in Figures 1 and 2. Here the entire area in view was treated for 15 minutes with 0.1 M lactate buffer–1 M sucrose, pH 4.5. (Approx. mag. ×2330.)

FIG. 4.—Tooth 11. Standard surface, produced as described in text, treated with 1 M sucrose solution adjusted to pH 11 with sodium hydroxide. (Approx. mag. ×3380.)

scopic examination of this particular replica, for example, showed the smooth regular appearance typical of an unetched surface on one side of the boundary and the rough irregular appearance typical of etching on the other side. Figures 2 and 3 provide additional views of the same replica. In Figure 2 the entire area in view was in the region treated with the lactate buffer, whereas the area shown in Figure 3 had been treated with the lactate-buffer–sucrose solution. These figures show that, although both solutions had a pH 4.5, their actions were not equal. The same result was obtained in other teeth; nothing would be gained by additional illustrations of the results obtained in other teeth by the same treatment.

When the etching actions of 0.1 M lactate buffer, pH 4.5, and pH 4.5 lactate buffer containing 1 M sodium chloride were compared, no difference was found. Both portions of the enamel surface exhibited the etched appearance of the type shown in Figure 2. Sodium chloride, therefore, exerts no retarding effect on the etching of enamel by lactate buffer. The use of glucose in place of sucrose results in some retardation of acid etching. Thus when 0.1 M lactate buffer–1 M glucose solution, pH 4.5, is compared with the regular 0.1 M lactate buffer, pH 4.5, the glucose-containing solution causes less etching. The difference appears to be much less than that shown by sucrose.

Neutral sucrose solutions caused no noticeable change in the enamel surface in the relatively short period of 15 minutes; however, at pH 11 sucrose did attack the enamel surface. That is, 1 M sucrose solution adjusted to pH 7 with a very small amount of sodium hydroxide had no noticeable effect on the tooth surface, whereas when the sucrose solution was raised to pH 11 with sodium hydroxide the resulting solution produced a slight but detectable change in the enamel surface in 15 minutes. The surface was roughened slightly, as shown in Figure 4. Additional work is required to investigate the nature of this action of sucrose on enamel in basic solutions.

DISCUSSION

When it was found previously that sucrose lowered the dissolution rate of powdered enamel particles in lactate-buffer solution,[1] several possible explanations were offered. They included viscosity effects, coating effects, chelation or complexing effects, and hydration effects. Although the present work provides no actual direct proof that any of these effects is operating, the fact that the present results do agree with those from the initial study increases the possibility that such effects do operate. For example, in the original discussion[1] it was suggested that at least part of the observed sucrose reduction of enamel solubility in acid might be due to the nature of the test. That is, small enamel particles agitated in a container might possibly act differently from intact enamel surfaces treated with the same solutions. As it developed, the effect is also observed in the intact enamel surface. The discussion offered in the previous article,[1] therefore, applies to the present work.

Although more work with other solutes substituted for sucrose will be required to delineate it, the etch-depressant action of sucrose appears to have some specificity. Certainly, the action is not unique, as shown by the slight effect found for glucose. That more is required, however, than the simple presence of a 1 M concentration of solute is indicated by the inactivity of sodium chloride. Purely from the practical standpoint of most direct concern in dental caries, of course, it would not matter if

other substances were found to be equally as effective as sucrose. The important point is that sucrose is frequently present in the plaque on the tooth surfaces in reasonably high concentrations and thus is available to exert its effect. Furthermore, because it is an important substrate for acid formation, sucrose is in the plaque at the time when acid concentration becomes appreciable. From a theoretical standpoint it is important to determine whether other substances may also exert actions similar to that of sucrose, even though those substances are in fact never found on the tooth surface in vivo. More work along this avenue may provide information sufficient to establish the mode of action of sucrose.

Additional work is also required to determine the nature of the action of sucrose on enamel in basic solutions. It appears reasonable to assume that sucrose-calcium chelation compounds will prove to be of importance in this case. It also appears possible that differential etching of the enamel surface by basic sucrose solutions may provide an additional approach to the study of enamel structure.

Finally, it will be of interest to determine (1) whether teeth from caries-active individuals etch more rapidly in lactate buffer than do teeth from individuals with no caries and (2) whether sucrose depresses the lactate enamel-etching rate more in one group than in the other.

SUMMARY

In confirmation of earlier chemical studies, sucrose has been shown by electron microscopy to exert a retarding action on the rate at which pH 4.5 lactate buffer etches human dental enamel surfaces. Further work is necessary to ascertain the nature of the action.

These results should not be construed as an argument against the accepted role of sucrose as a fermentation substrate for acid formation on the tooth surface. Instead, they should be regarded as additional evidence for the existence of multiple factors involved in caries development.

REFERENCES

1. Blackwell, R. Q., and Fosdick, L. S. *J. D. Res.*, **35**:210, 1956.
2. Fosdick, L. S., McMillan, L., and Blackwell, R. Q. *Arch. Oral Biol.*, **1**:211, 1959.

Studies on Periodontal Disease in the Mouse
II. Genetic and Maternal Effects

PAUL N. BAER, LYMAN B. CRITTENDEN, GEORGE E. JAY, JR., and JACOB E. LIEBERMAN

National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland

In recent publications from our laboratory, we have been able to show that strain differences in resistance or susceptibility to periodontal disease exist in inbred mice.[1, 2] The inbred strain STR/N, for example, has been found to be susceptible, while DBA/2JN is quite resistant. This investigation was initiated to determine the possible effects that genetic factors might have in the etiology of periodontal disease. A search of the literature failed to reveal any publications on this subject.

MATERIALS AND METHODS

"S" will represent the STR/N line and "D" the DBA/2JN line throughout the remainder of this paper. The following crosses and backcrosses were made (Tables 1, 2, 3, and 4); S (females) $\times$ D (males) and D (females) $\times$ S (males) resulting in 114 S $\times$ D F_1 and 103 D $\times$ S F_1 mice. The F_1 generations were then bred together to produce 227 S $\times$ D F_2 and 208 D $\times$ S F_2 mice. Four backcross generations were also completed, which yielded 112 S(S $\times$ D), 121 (S $\times$ D)D, 42 (D $\times$ S)S, and 24 D(D $\times$ S) mice.

The mice were weaned at approximately 30 days of age. Half the animals in each generation were fed Purina Laboratory chow and the other half a high-fat diet (diet 10,046, 57 per cent vegetable oil).[1] All the mice were sacrificed at 170 days of age, defleshed by the papain method, and three mandibular teeth and three maxillary teeth were scored for alveolar bone loss.[1] The two diets were used because of indications from previous work done in our laboratory that diet differences existed. The entire experimental period lasted 529 days, and a total of 1,156 animals was used.

The total scores for the maxilla and mandible were used for the analysis. Ridits were not substituted for periodontal scores as in a previous paper[2] because the totaling of the three scores tends to normalize the distribution and equalize the intervals between total scores. The use of a total score thus removed some of the undesirable statistical properties associated with the original scores of the individual teeth. Thus the score for each jaw is one of the integers from 0 to 12.

The term "generations" used throughout this paper refers to the original parents, as well as to the progeny of all the crosses. The reciprocal crosses are considered to be different generations for the present purposes.

In order to check the possibility that contagion is a major factor in the difference

Received for publication November 20, 1959; revised by authors August 30, 1960.

observed between these two strains, a separate experiment was set up in which 3 S and 3 D mice were housed together in each of four cages from about 30–170 days of age.

RESULTS AND ANALYSIS

Tables 1, 2, 3, and 4 give the distributions and average total scores of periodontal involvement for each jaw by generation and sex within diet. It may be noted that mice fed the high-fat diet were scored lower for both the maxilla and the mandible, the difference being more striking for the maxilla. Tables 1, 2, 3, and 4 also show a slight tendency for females to show more involvement than males.

The analysis of variance presented in Table 5 was used to investigate the sources of variation in these data. The two backcrosses in which only a small number of mice were available were omitted from the analysis, leaving 64 subgroups whose means were available for analysis. An unweighted-mean analysis of variance was used[3] because there were unequal numbers of litters and mice in each subgroup. The proper error term for testing the main effects and interactions which involve comparisons of different levels of treatment on different litters and different mice is the between-litter mean square, and the proper error term for testing the differences between the

TABLE 1

DISTRIBUTION OF TOTAL MAXILLARY SCORES, BY GENERATION, SEX, AND DIET (STOCK DIET)

Generation		Sex	Total Maxillary Score													No.	Mean
			0	1	2	3	4	5	6	7	8	9	10	11	12		
P	S	M	....	....	....	....	....	6	12	2	7	1	3	1	....	32	6.94
		F	....	....	....	....	....	1	6	15	2	4	1	....	....	29	7.17
	D	M	....	3	6	5	2	2	1	....	....	....	....	....	....	19	2.84
		F	....	1	3	5	5	4	....	....	....	....	....	....	....	18	3.44
F_1	S×D	M	....	....	2	5	8	10	5	....	....	....	....	....	....	30	4.37
		F	....	....	....	6	8	3	5	2	....	....	....	....	....	24	4.54
	D×S	M	....	....	5	3	2	9	6	....	....	....	....	....	....	25	4.32
		F	....	....	1	3	6	12	5	....	....	....	....	....	....	27	4.63
F_2	S×D	M	....	1	4	10	14	22	1	2	....	2	....	....	....	56	4.34
		F	....	3	6	4	10	25	10	1	....	1	....	....	....	60	4.47
	D×S	M	1	7	14	13	9	7	2	....	....	....	....	....	....	53	2.96
		F	....	7	11	15	6	11	1	....	....	....	....	....	....	51	3.12
BC	S(S×D)	M	....	....	....	1	1	11	9	8	....	....	....	....	....	30	5.73
		F	....	....	....	1	1	15	4	7	....	....	....	....	....	28	5.54
	(S×D)D	M	....	1	9	5	4	10	....	2	....	....	....	....	....	31	3.68
		F	....	2	5	5	5	8	....	3	1	....	....	....	....	29	4.00
	(D×S)S	M	....	1	2	....	4	6	....	....	....	....	....	....	....	13	3.92
		F	....	....	2	2	2	2	....	....	....	....	....	....	....	8	3.50
	D(D×S)	M	....	1	....	....	3	....	....	....	....	....	....	....	....	4	3.25
		F	....	2	3	1	3	....	....	....	....	....	....	....	....	9	2.56

two jaws, which can be measured on the same mice, and the interactions with jaws is the jaw-by-litter mean square.

The between-litter, within-litter, jaw-by-litter, and jaw-by-within-litter analysis was performed in order to obtain the proper error terms for testing the differences between the means and also to obtain estimates of the relative importance of the above sources of variation. The pooled analysis was used, in spite of the fact that the variances appear to be heterogeneous, as expected, because the higher and lower scores are expected to show a lowered variance as they approach the limits of 12 and 0. Tests of heterogeneity of the between-litter variances are not highly significant, showing that the heterogeneity is not extreme. These data suggest a positive association between the mean and the variance, probably because most of the means fall in the lower part of the 0 through 12 scale.

The F tests show that generation, diet, and sex have a significant effect on total periodontal score. The four significant two-factor interactions are of some interest. The diet-by-generation interaction appears to be caused by a wider range of generation means shown by mice fed the high-fat diet. Also the S(S × D) backcross was exceptional, for it showed a higher score on the high-fat diet, contrary to the average

TABLE 2

DISTRIBUTION OF TOTAL MAXILLARY SCORES, BY GENERATION, SEX, AND DIET (DIET 10,046)

Generation		Sex	Total Maxillary Score													No.	Mean
			0	1	2	3	4	5	6	7	8	9	10	11	12		
P	S	M	....	....	1	....	....	5	7	10	1	2	....	....	....	26	6.35
		F	....	....	....	1	1	4	6	10	1	2	3	....	....	28	6.75
	D	M	3	11	9	2	1	....	....	....	....	....	....	....	....	26	1.50
		F	2	13	11	1	....	....	....	....	....	....	....	....	....	27	1.41
F_1	S×D	M	....	....	6	7	14	2	1	1	....	....	....	....	....	31	3.61
		F	....	3	7	4	2	3	7	3	....	....	....	....	....	29	3.97
	D×S	M	....	2	7	11	5	1	....	1	....	....	....	....	....	27	3.00
		F	....	1	11	8	2	....	2	....	....	....	....	....	....	24	2.79
F_2	S×D	M	1	19	18	6	6	3	2	....	....	....	....	....	....	55	2.25
		F	....	15	10	14	5	4	4	4	....	....	....	....	....	56	3.02
	D×S	M	17	29	3	2	1	....	....	....	....	....	....	....	....	52	0.87
		F	11	34	3	4	....	....	....	....	....	....	....	....	....	52	1.00
BC	S(S×D)	M	....	1	....	....	....	10	9	5	4	....	1	....	....	30	6.07
		F	....	1	1	....	3	4	6	6	3	....	....	....	....	24	5.71
	(S×D)D	M	2	18	4	7	....	....	....	....	....	....	....	....	....	31	1.52
		F	....	14	7	3	4	2	....	....	....	....	....	....	....	30	2.10
	(D×S)S	M	1	5	4	....	....	....	....	....	....	....	....	....	....	10	1.30
		F	....	7	4	....	....	....	....	....	....	....	....	....	....	11	1.36
	D(D×S)	M	....	3	1	....	....	....	....	....	....	....	....	....	....	4	1.25
		F	....	5	2	....	....	....	....	....	....	....	....	....	....	7	1.29

effect of that diet. The interactions with jaw do not involve definite switches in rank; rather, the reciprocal generation differences appear to be greater in the maxilla, accounting for part of the jaw-by-generation interaction. The jaw-by-diet and jaw-by-sex interactions may be explained in a similar way. These interactions do not necessarily indicate true biological interaction. Therefore, the qualitative response on both diets and measurements on both jaws are the same, and the interactions observed only indicate a quantitative deviation from an additive scheme, probably due to the scale used.

The pooled analysis of variance within generation-sex-diet groups shows that the variances contributed by litters and by the jaw-by-litter interaction are significant. The following estimates of components of the total within-group variance may be attributed to the four variables of classification after correcting for the fixed effects of the jaws: Between-litters, 0.44 ($\hat{\sigma}^2_B$); within-litters, 1.32 ($\hat{\sigma}^2_W$): jaw-by-between-litters, 0.20 ($\hat{\sigma}^2_{JB}$); and jaw-by-within-litters, 0.73 ($\hat{\sigma}^2_{JW}$). The above figures show that a relatively small but significant proportion of the variance may be attributed to litter differences and to inconsistencies resulting from measuring both jaws, while the

TABLE 3

DISTRIBUTION OF TOTAL MANDIBULAR SCORE BY GENERATION, SEX, AND DIET (STOCK DIET)

Generation	Sex	Total Mandibular Score															No.	Mean
		0	1	1.5*	2	3	4	4.5*	5	6	7	8	9	10	11	12		
P S	M						1		3	9	6	5	2	2	4		32	7.41
	F								1	2	20	3	1	1	1		29	7.28
P D	M		2		8	8	1										19	2.42
	F		1	1	6	10											18	2.47
F_1 S×D	M				1	4	10		11	4							30	4.43
	F					7	8		7		1	1					24	4.29
F_1 D×S	M		4		8	8	3		2								25	2.64
	F		1		4	4	7		8	3							27	3.96
F_2 S×D	M		7		9	10	11		13	3			1		2		56	3.80
	F		5		5	15	11		13	6	3	1	1				60	4.05
F_2 D×S	M		12		21	15	3		1	1							53	2.30
	F		10		16	11	8		3	2		1					51	2.78
BC S(S×D)	M						6		13	6	2		1	1	1		30	5.63
	F						3		9	11	4	1					28	5.68
BC (S×D)D	M		4		9	5	8		4					1			31	3.19
	F		3		4	8	6			6	1				1		29	3.90
BC (D×S)S	M				3	6	3		1								13	3.15
	F		2		1	1	4										8	2.88
BC D(D×S)	M	1	1		1		1										4	1.75
	F	1	4		2		1		1								9	1.89

* In a few cases, the D strain had impacted or missing mandibular teeth, so the other two teeth were averaged to obtain a score for the third tooth.

TABLE 4

Distribution of Total Mandibular Score by Generation, Sex, and Diet (Diet 10,046)

Generation	Sex	Total Mandibular Score															No.	Mean
		0	1	1.5*	2	3	4	4.5*	5	6	7	8	9	10	11	12		
P	S	M	...	...	...	...	...	3	...	3	9	1	5	2	2	1	...	26	6.81
		F	...	...	...	...	...	1	...	2	4	12	3	1	1	3	1	28	7.50
	D	M	2	8	1	12	3	...	...	...	...	...	...	...	...	...	...	26	1.63
		F	...	6	2	11	7	...	...	1	...	...	...	...	...	...	...	27	2.11
F_1	S×D	M	...	1	...	11	4	5	...	6	3	1	...	...	...	...	...	31	3.55
		F	...	1	...	2	1	7	1	8	6	2	1	...	...	...	...	29	4.78
	D×S	M	...	6	...	17	2	2	...	...	...	...	...	...	...	...	...	27	2.00
		F	...	2	...	14	5	1	...	1	1	...	...	...	...	...	...	24	2.50
F_2	S×D	M	...	13	...	11	12	10	...	3	5	1	...	...	...	...	...	55	2.96
		F	...	9	...	4	10	11	...	12	2	3	4	1	...	...	...	56	4.02
	D×S	M	3	26	...	12	5	1	...	2	1	1	1	...	...	...	...	52	1.92
		F	2	30	...	13	4	3	...	...	...	...	...	...	...	...	...	52	1.54
BC	S(S×D)	M	...	...	...	1	...	...	...	13	3	3	3	5	2	...	...	30	6.50
		F	...	...	...	1	...	3	...	4	6	3	2	3	2	...	...	24	6.42
	(S×D)D	M	...	16	...	10	1	3	...	1	...	...	...	...	...	...	...	31	1.81
		F	...	4	...	14	6	4	...	1	...	1	...	...	...	...	...	30	2.60
	(D×S)S	M	...	2	...	6	1	...	...	1	...	...	...	...	...	...	...	10	2.20
		F	...	5	...	3	3	...	...	...	...	...	...	...	...	...	...	11	1.82
	D(D×S)	M	...	3	...	1	...	...	...	...	...	...	...	...	...	...	...	4	1.25
		F	...	3	...	2	1	1	...	...	...	...	...	...	...	...	...	7	2.00

* In a few cases, the D strain had impacted or missing mandibular teeth, so the other two teeth were averaged to obtain a score for the third tooth.

TABLE 5

Analysis of Variance of 64 Unweighted Group Means and Within-Group Analysis Used for Testing Group Means

Sources	Analysis of Variance d.f.	Mean Squares	*F*
Generations	7	24.88	130.9*
Diets	1	11.84	62.3*
Generations×diets	7	1.05	5.5*
Sex	1	1.69	8.9*
Sex×generations	7	0.13	<1
Sex×diets	1	0.03	<1
Sex×generations×diets	7	0.14	<1
Litters	265	0.19	2.1*
Within-litters	793	0.09	.....
Jaw	1	0.01	<1
Jaw×generations	7	0.35	7.0*
Jaw×diet	1	2.56	51.2*
Jaw×sex	1	0.22	4.4†
Residual	22	0.06	1.2
Jaw×litters	265	0.05	2.5*
Jaw×within-litters	793	0.02	.....

* Significant at the 0.01 probability level.

† Significant at the 0.05 probability level.

large proportion of this variance is due to variance within litters and within jaw-litter groups. Litter differences may result from a large number of environmental and perhaps genetic differences between litters. Genetic litter differences should not be an important factor in this experiment because the two parental strains are presumably nearly homozygous. Environmental litter differences in an etiologically complex disease can arise because of differences in the female parent, such as age, number of previous litters, disease conditions, and general health during pregnancy and lactation, as well as differences in litter size, litter environment other than maternal environment, and to differences associated with the different dates at which the litters were raised and scored. However, the proportion of the total variance attributable to litter means is relatively small compared with some traits such as 60-day body weights.[4]

The variance components estimated from the pooled within-generation-sex-diet group analysis give information which is important for the planning of future experiments. The most practical experiment is the one which reduces the variance of the group mean as much as possible with the least amount of time and space allocated to the experiment. If we assume that the same variable is in reality being measured in both jaws except for scale and sampling errors, we can set up a formula for estimating the variance of a treatment mean, assuming that the variance components we have estimated are representative of the experiments planned. This formula is

$$\hat{\sigma}^2_{\bar{X}} = \frac{\hat{\sigma}^2_B}{l} + \frac{\hat{\sigma}^2_W}{ln} + \frac{\hat{\sigma}^2_{JB}}{jl} + \frac{\hat{\sigma}^2_{JW}}{jln},$$

where $\hat{\sigma}^2_{\bar{X}}$ is the variance of the group mean; $\hat{\sigma}^2_B$ is the between-litter variance; $\hat{\sigma}^2_W$ is the within-litter variance; $\hat{\sigma}^2_{JB}$ is the jaw-by-between-litter variance; $\hat{\sigma}^2_{JW}$ is the jaw-by-within-litter variance; l is the number of litters per group; n is the number of mice per litter; and j is the number of jaws measured. Using the above formula, one may plan experiments substituting different sample sizes and arrive at an approximation of the variance of the treatment group means which fits the experimental facilities and the size of the differences that are of interest to the investigator. If one were planning to measure one jaw only, probably the maxilla should be chosen because there is an indication from these data that the within-litter variance is lower for this jaw.

Genetic interpretation of the generation means.—Table 4 gives the generation means and standard errors for the average maxillary and mandibular scores averaged over jaws and sex presented for each diet. Two factors make these means difficult to intepret. The first is the necessity of using a subjective score which is difficult to transform to an appropriate scale for genetic analysis, and the second is the fact that the various generations were not scored over comparable periods of time and no effort was made to make the ages of the parents comparable in each group. Because the D strain did not reproduce as well as the S strain, animals were scored over a longer period when the D genetic material entered from the female side. However, the time trends observed were not important enough to account for the consistent reciprocal effects to be discussed. Also, the between-litter variances were not consistently greater for the groups scored over the longer periods of time.

The means of the generations for each diet are presented diagramatically in Figure 1. The generations are grouped on the horizontal axis by the proportion of non-sex-linked

genetic material they have in common with the parental generations (P); thus the F_1 and F_2 generations have an equal contribution from the two strains and the backcrosses (BC) have three-fourths of the genetic material of one and one-fourth of the other, depending on which way the backcross was made. The vertical scale is the average total score for each generation. If a line connected the true values of the parental generations, it would indicate where the average should fall if the genes involved had completely additive effects on the phenotypes. Additivity would hold if the observed means were perfectly associated with the amount of non-sex-linked genetic material contributed by each parental strain. Since the means of the parental generations are estimates, two dotted lines connect the points two standard errors above and below the parental means, to give a rough idea of the region in which the true line is located. Deviations from a straight line may be due to the inadequacy of the scale, sampling errors, non-additive effects of genes, parental effects, and non-random environmental factors. The most striking feature of this figure is that generations which theoretically have an identical chromosomal makeup show differences many of which are large compared with the standard errors of their means given in

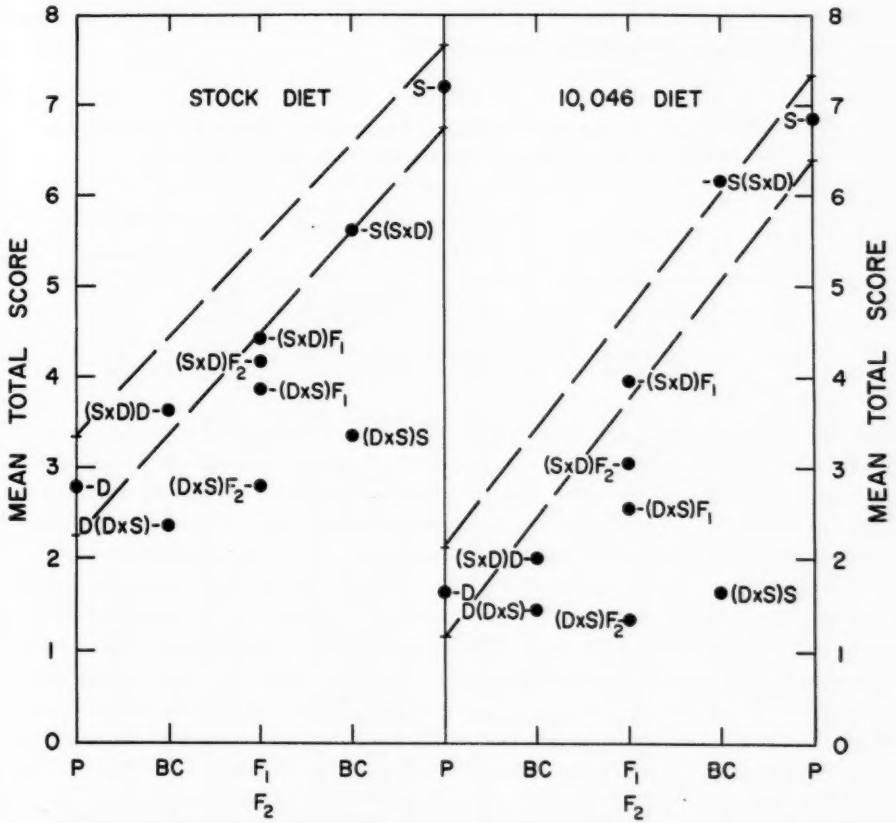


FIG. 1.—Observed generation mean periodontal scores for each diet. *S* = STR/N mice; *D* = DBA/2JN mice; *P* = original pattern strains; *BC* = backcross.

Table 6. Considering each type of cross separately, a reciprocal difference is observed in every case. In each case the cross more closely resembles the parental line that entered from the female side.

Sex linkage may be eliminated as a major cause of these reciprocal effects because a reciprocal difference should be observed only in the males in the F_1, because the males are heterogametic. Moreover, males should more closely resemble their female parents than the females. No striking differences of this type were observed, nor was the sex-by-generation interaction found to be significant in Table 5, as it should be if strong sex linkage were present.

These considerations lead one to a maternal extra-chromosomal explanation. Reciprocal F_1 differences may be explained on the basis of a direct effect of the different maternal genotypes. However, F_2 reciprocal differences cannot be explained in this way because the genetic makeup is theoretically identical in the reciprocal parents of the F_2 generations. The backcrosses confirm the hypothesis of maternal effects, but the choice of matings makes it impossible to confirm the evidence, given by the F_2 crosses, that the effect is independent of the maternal genotype. The evidence suggests that, since the reciprocal effects carry over to the F_2 generations, one or both of the original strains has an extra-chromosomal influence which is passed on for at least two generations through the female parent.

TABLE 6

MEANS, STANDARD ERRORS, AND WITHIN-LITTER VARIANCES FOR EACH GENERATION WITHIN EACH DIET (MAXILLA AND MANDIBLE COMBINED)

Diet	Generation	No. of Mice	Mean ± Standard Error	Within-Litter Variance	Coefficient of Variation
Stock	P S	61	7.20±0.22	2.00	20
	P D	37	2.79±0.28	0.64	29
	F_1 S×D	54	4.41±0.23	1.06	23
	F_1 D×S	52	3.89±0.24	1.40	30
	F_2 S×D	116	4.16±0.16	2.09	35
	F_2 D×S	104	2.79±0.17	1.21	39
	BC S(S×D)	58	5.64±0.22	1.29	20
	BC (S×D)D	60	3.64±0.22	2.36	42
	BC (D×S)S	21	3.36±0.37	.....	.....
	BC D(D×S)	13	2.36±0.47	.....	.....
10,046	P S	54	6.85±0.23	2.54	23
	P D	53	1.66±0.23	0.60	46
	F_1 S×D	60	3.98±0.22	2.08	36
	F_1 D×S	51	2.57±0.24	0.98	38
	F_2 S×D	111	3.06±0.16	2.53	52
	F_2 D×S	104	1.33±0.17	1.08	78
	BC S(S×D)	54	6.18±0.23	3.22	29
	BC (S×D)D	61	2.01±0.22	0.94	48
	BC (D×S)S	21	1.67±0.37	.....	.....
	BC D(D×S)	11	1.45±0.51	.....	.....

The reciprocal differences just discussed are emphasized because they are unexpected from the classical genetic point of view. They should not hide the fact that the means, especially of the generation where the S strain enters as the female parent, follow the general trend predicted from a genetic interpretation. That is, both maternal and chromosomal factors may be operating simultaneously.

Genetic interpretation of the variances.—If the S and D strains were completely homozygous, the parental and F_1 variances would be entirely environmental, and the F_2 and backcross (BC) generations should show higher variances due to the segregation of genetic material which is different in the two original strains. Within-litter variances are presented in Table 6 for purposes of comparison. All the genetic segregation should occur within litters because the parents of each type of cross should be genetically identical. Thus environmental variance associated with litter

TABLE 7

TOTAL MAXILLARY AND MANDIBULAR SCORES FOR S AND D MICE CAGED TOGETHER FROM WEANING TO 170 DAYS OF AGE

Cage	Strains	No. of Animals	Total Scores*
1.....	S	3	38
	D	3	5
2.....	S	3	29
	D	3	6
3.....	S	3	41
	D	3	2
4.....	S	3	34
	D	3	4

* The maximum individual score possible (most severe periodontal disease) is 24. Thus the maximum possible score for each strain per cage was 72.

means is removed from the variances to be compared. The apparent association between the means and the within-litter variances make comparisons difficult. However, comparison of the parental (P) and F_1 variances with the F_2 and backcross (BC) variances fail to show any striking evidence of genetic segregation. When the within-litter coefficients of variation[1] are investigated, there is some suggestion that the F_2 crosses are more variable than the F_1.

The variances do not suggest that the S and D strains differ at a small number of genetic loci having a large effect on periodontal disease but rather that this disease is influenced by a large number of loci, each having a small effect, and by considerable uncontrolled environmental variation.

Infection through contact.—Table 7 presents data from a separate experiment which shows that infection through the contact of mice from 30 to 170 days of age is not an important factor in determining the differences in periodontal disease expressed by the S and D strains.

DISCUSSION

Unexpected differences in reciprocal crosses of inbred lines and species hybrids were reported early in the literature of genetics. Most of these differences have been found in plants, lower animals, and micro-organisms.[5] In general, these have been found to be maternal effects because the female parent contributes more cytoplasm to the zygote and, at least in mammals, she has more contact with the young during development than the male. Some of these differences have been found to be under the direct control of the maternal genotype, and the reciprocal effect disappears in the F_2 generation because the reciprocal maternal parents of this generation have the same chromosomal makeup. Other examples of reciprocal differences have persisted through several generations of intercrossing and backcrossing, and it must be concluded that some agent causing the effect is being passed on through the mother independent of her genetic makeup. Evidently, the case under consideration is of the latter type because the reciprocal difference persists into the F_2.

In mice the female parent has several opportunities to affect the development of her offspring other than through the chromosomes she contributes. She may influence them through the extra-chromosomal material of the ovum, the intra-uterine environment she provides, or through her milk.

One of the most extensively studied cases of maternal influence in mice is the mammary tumor agent, which is a virus-like particle passed on to the young through the milk. Strains of mice having a very high incidence of mammary tumors have been shown to carry this agent. However, low-incidence strains may or may not have it. The present evidence suggests that both the susceptibility of the mice independent of the agent and the agent itself may be influenced by genes.[6, 7]

In some cases high- and low-incidence leukemia strains of mice behave differently in reciprocal crosses. It is suggested that some protective agent is passed on through the female parent, and again there is evidence that genes have an effect independent of the maternal influence. Evidence is conflicting as to the mode of transmission of this agent.[7]

Another example of a reciprocal difference which, however, was not passed on to the F_2 was reported by Green and Russell.[8] They found reciprocal differences in F_1 crosses of two lines of mice which differed in the number of lumbar vertebrae present.

Brumby[9] reports reciprocal differences in body weight in crosses of large and small strains of mice. Foster nursing and transplantation of ova suggest complex interactions between the genotype of the zygote and the prenatal and postnatal influence of the mother.

The maternal influence in the lines discussed in this paper is evidently largely independent of the maternal genotype. Whether this is an influence promoting periodontal disease carried by the S strain or a protective effect of the D strain cannot be determined from the present data. This question and the problem of mode of transmission must be investigated in future experiments involving foster nursing and the transplantation of ova or ovaries.

CONCLUSIONS

The STR/N and DBA/2JN lines of mice evidently differ at a large number of genetic loci, each of which has a small effect on periodontal disease.

The two strains differ by some maternal extra-chromosomal influence which may be passed on for at least two generations.

The extreme differences between the means of the two pure strains are due to both genetic and extra-chromosomal maternal factors.

There is considerable uncontrolled environmental variation in the expression of periodontal disease.

Mice fed the high-fat diet 10,046 show significantly less periodontal involvement than mice fed the stock diet.

Females show slightly more periodontal involvement than males.

There is no significant difference between the mean mandibular and maxillary scores.

Infection by the mutual contact of mice is not a major factor in the difference in periodontal disease observed between these two strains.

REFERENCES

1. Baer, P. N., and Lieberman, J. E. Observations on Some Genetic Characteristics of the Periodontium in Three Strains of Inbred Mice, *Oral Surg., Oral Path., Oral Med.*, **12**:820, 1959.
2. ———. Periodontal Disease in Six Strains of Inbred Mice. *J. D. Res.*, **39**:215, 1960.
3. Anderson, R. L., and Bancroft, T. A. *Statistical Theory in Research*, New York: McGraw-Hill Book Co., Inc., 1952.
4. Chai, C. K. Analysis of Quantitative Inheritance of Body Size in Mice. II. Gene Action and Segregation, *Genetics*, **41**:165, 1956.
5. Caspari, Ernst. Cytoplasmic Inheritance. In *Advances in Genet.*, Vol. **2.** New York: Academic Press, 1948.
6. Bittner, J. J. Genetic Concepts in Mammary Cancer in Mice. In Genetic Concept for the Origin of Cancer, *Ann. N.Y. Acad. Sci.*, **71**:943, September, 1958.
7. Law, L. W. Genetic Studies in Experimental Cancer. In *Advances in Cancer Research*, Vol. **2.** New York: Academic Press, 1954.
8. Green, E. L., and Russell, W. L. A Difference in Skeletal Type between Reciprocal Hybrids of Two Inbred Strains of Mice (C57BLK and C3H), *Genetics*, **36**:641, 1951.
9. Brumby, P. J. The Influence of the Maternal Environment on Growth in Mice, *Heredity*, **14**:1, 1960.

Relationship of Tooth Size to Cusp Number and Groove Conformation of Occlusal Surface Patterns of Lower Molar Teeth

ALBERT A. DAHLBERG

Zoller Memorial Dental Clinic and Department of Anthropology, University of Chicago

Lower molar occlusal surface pattern variation has always been assumed to be correlated with size, particularly the aspect concerning the number of cusps. Although it is not necessary to show a statistically significant relationship in every instance of comparison, such a consideration of size and pattern is helpful in clarifying and evaluating the general evolutionary trends occurring in the permanent lower molar teeth of modern hominid populations.

EXPERIMENTAL METHODS

In comparing similarly patterned homologous teeth of different population groups, it has been found that the measurements are greater in one group than in another. The mean mesiodistal measurement of four-cusped lower molar teeth of modern Iraqis, for example, is much smaller than that for New Britain Melanesians or Australian aborigines (Table 1). Therefore, to make the comparison meaningful, the four-cusped teeth must be compared with the five-cusped teeth within the same population group.

Since Hellman[1] in 1928 outlined the various occlusal surface patterns and discussed their frequency in differing population groups there have been many other studies and discussions of these phenomena. Hellman's original classification of lower molar occlusal surfaces was based on the number of cusps and the groove patterns established between them. The basic pattern is the Y5 type known as "dryopithecoid," the "5" referring to the main cusps which are homologous with the major cusps of previous tooth forms in phylogeny. The four-cusped class includes all these cusps except the hypoconulid on the distal aspect, which is the most variable one on the occlusal surfaces. The number 4 or 5 is combined with the letter Y or a plus sign (+) which indicates the arrangement of the grooves between these cusps.

The +4 pattern is considered to be the most evolutionarily advanced type, and the Y5 the ancestral variety. Thus the +4 pattern is recognized as being further removed from the primitive type. The fact that the +4 tooth is a smaller one than the Y5 also follows the general trends of reduction of dental components in the modern forms. Jorgensen[2] in 1955 proposed an additional groove conformation which he termed the X pattern. This X category, though desirable for most studies, was not available for the present report.

This study was supported in part by Research Grant D-165 from the N.I.D.R., U.S. Public Health Service.

Received for publication January 12, 1960; revised by author August 30, 1960.

TABLE 1*

LENGTH (MESIODISTAL) MEASUREMENTS (IN MILLIMETERS) OF LOWER PERMANENT MOLAR TOOTH CROWNS BY OCCLUSAL SURFACE CUSP-GROOVE PATTERNS

		M1			M2				M3			
	Y5	Y4	+5	+4	Y5	Y4	+5	+4	Y5	Y4	+5	+4
New Britain Melanesian..	11.7±0.04	11.4±0.46	11.7±0.31	11.3	11.9	10.9±0.1	11.8±0.13	11.0±0.04	11.5±0.17	10.8±0.07	11.5±0.07	10.6±0.12
Arab (Baghdad).....	11.2±0.17	.....	.....	.....	.....	.....	.....	10.6±0.16	.....	.....	.....	10.0±0.18

* The categories of cusp-groove patterns are designated by either a letter Y or a + sign combined with the number 4 or 5, the number of cusps. The Y and + indicate the pattern of grooves formed between the cusps. The mean measurements were derived from 205 Melanesian and 25 Arab individuals in the skeletal collections of the Department of Anthropology, Chicago Natural History Museum.

RESULTS

Over 200 dentitions of the Chicago Natural History Museum Melanesian collection were involved in this study. Table 2 shows the distribution of the various occlusal surface patterns with the means, number of cases, standard deviations, coefficients of variability, and standard errors of the means for the two measurements, length and breadth, which were made on the Melanesians. Inspection of the tables shows what seems to be a rather obvious larger size for the teeth of the five-cusped variety. It would not be expected that there would be much variation in breadth of these varying patterned teeth because the difference in the reduction of number of cusps involves the other dimension of the tooth, that is, the length (mesiodistal). This is confirmed by the data shown. The breadth does not vary much between the Y5 and the +4 pattern in each of the three teeth. However, the difference is much greater for the length measurements.

Table 3 pools the different patterns for all three teeth according to cusp number. Since M1, M2, and M3 were measured on each subject, where possible, the data for

TABLE 2*

LENGTH AND BREADTH MEASUREMENTS OF LOWER MOLAR TOOTH CROWNS OF NEW BRITAIN MELANESIANS

	LENGTH						BREADTH					
	M	*N*	σ	*V*	σ_M	*R*	*M*	*N*	σ	*V*	σ_M	*R*
	M1						M1					
Y5	11.67	205	0.55 ±0.027	4.7 ±0.233	0.0384	0.4	10.9	201	5.15 ±0.026	4.7 ±0.234	0.0363	0.6
+5	11.7	5	0.625±0.22	5.3 ±1.9	0.312	.....	11.1	5	0.60 ±0.21	5.42±1.9	3.0	....
Y4	11.4	6	1.03 ±0.33	9 ±2.84	0.46	.....	10.7	6	1.00 ±0.316	9.0 ±2.84	0.447	....
+4	11.3	2	.....	.....	.....	.....	10.55	2	.....	.....	.....	....
	M2						M2					
Y5	11.9	1	σ	.....	.....	1.0	11.5	1	.....	.....	.....	...
+5	11.8	25	0.64 ±0.09	5.4 ±0.76	0.128	.....	11.0	25	0.495±0.07	4.5 ±0.63	0.099	1.1
Y4	10.9	23	0.503±0.074	4.6 ±0.66	0.105	.....	10.4	26	0.485±0.67	4.65±0.645	0.095	....
+4	11.0	139	0.45 ±0.027	4.1 ±0.246	0.0382	.....	10.4	139	0.54 ±0.032	5.2 ±0.310	0.046	....
	M3						M3					
Y5	11.54	15	0.65 ±0.118	5.65±1.03	0.168	0.960	10.5	13	0.678±0.138	6.45±1.32	0.196	0.5
+5	11.5	101	0.71 ±0.05	6.6 ±0.434	0.071	.....	10.6	100	0.628±0.045	5.93±0.418	0.063	....
Y4	10.8	9	0.78 ±0.195	7.2 ±1.8	0.276	.....	10.5	9	0.70 ±0.18	6.7 ±1.7	0.246	....
+4	10.6	20	0.55 ±0.087	5.2 ±0.82	0.123	.....	10.1	21	0.665±0.102	6.6 ±1.01	0.145	....

* The statistical data of the various groove-cusp pattern categories of lower molar teeth are shown. *M* = mean, *N* = number, σ = standard deviation, *V* = coefficient of variation, σ_M = standard error of the mean, *R* = range.

TABLE 3

FOUR- AND FIVE-CUSPED LOWER MOLARS IN NEW BRITAIN MELANESIANS

	5 Cusps	4 Cusps
M1	210	8
M2	26	162
M3	116	29

the three teeth are dependent and cannot be pooled. The M1 data are insufficient to show differences between the four- and five-cusped teeth, since the sample contains only eight four-cusped teeth.

For M2 and M3 a two-by-two contingency table was made, dividing the four- and five-cusped teeth at the median of the combined group. A chi-square test was then done for both second and third molars (Tables 4 and 5), indicating differences be-

TABLE 4*

CONTINGENCY TABLE FOR DISTRIBUTION OF FOUR- AND FIVE-CUSPED MELANESIAN LOWER SECOND MOLARS

	5-Cusped		4-Cusped		
>11.0	Observed:	23	Observed:	63	85
	Expected:	11.76	Expected:	73.25	
≤11.0	Observed:	3	Observed:	100	103
	Expected:	14.25	Expected:	88.75	
		26		162	188

$$\Delta^2 = (\text{expected} - \text{observed})^2/\text{expected}$$

$$\chi^2 = \frac{126.45}{11.76} + \frac{126.45}{73.25} + \frac{126.45}{14.25} + \frac{126.45}{88.75}$$

$$= 10.76 + 1.73 + 8.88 + 1.42$$

$$= 22.79$$

* The contingency table for the chi-square test shows the arithmetic involved in determining the significance of the difference between median mesiodistal measurements of the four- and five-cusped lower second molars. The actually observed and the expected numbers in the categories form the basis of the computation. Δ^2 = (expected-observed)^2/expected. χ^2 = the sum of Δ^2s = chi-square.

Reference of the chi-square figures (22.79 for lower second molars, this table and 20.88 for lower third molars, Table 5) in a statistical table of chi-square under one degree of freedom gives a probability value of less than one in a thousand.

TABLE 5

CONTINGENCY TABLE FOR DISTRIBUTION OF FOUR- AND FIVE-CUSPED MELANESIAN LOWER THIRD MOLARS

	5-Cusped		4-Cusped		
>11.4	Observed:	67	Observed:	3	70
	Expected:	56	Expected:	14	
≤11.4	Observed:	49	Observed:	26	75
	Expected:	60	Expected:	15	
		116		29	145

$$\Delta^2 = (\text{expected} - \text{observed})^2/\text{expected}$$

$$\chi^2 = \frac{121}{56} + \frac{121}{14} + \frac{121}{60} + \frac{121}{15}$$

$$= 2.161 + 8.643 + 2.017 + 8.067$$

$$= 20.88$$

tween the four- and five-cusped teeth that would not occur once in a thousand times if samples of this size were taken at random from the population.

DISCUSSION

The trend in modern hominid dentitions has been toward a reduction in size and form of the teeth, as well as a retraction of the muzzle. The number of cusps and the associated pattern of grooves of lower molars have been widely discussed by students of dental evolution. It has been implied that reduced cusp number was accompanied by reduced size of the teeth. The question is whether or not the changes in morphology affect the size and dimensions of the teeth. Size and form could be independent qualities. The statistical analysis of these points in a series of lower molar teeth of the Melanesian population gives evidence that there is a relationship between the two elements. However, the sizal variation occurred in only one dimension, the mesiodistal and not in the buccolingual. This is not really unexpected, for the cusp involved in the reduction process is in the distal portion of the tooth and not in the buccolingual plane. The comparison of the mesiodistal length with the cusp number of the molar teeth showed this relationship to be statistically significant. Four-cusped molar teeth are smaller in their mesiodistal dimension than five-cusped teeth, assuming that they are from a population having a common genetic background.

SUMMARY

Statistical techniques were applied to data from over 200 Melanesian dentitions to determine the relationship between mesiodiostal dimension and number of cusps of the lower molar teeth.

REFERENCES

1. Hellman, Milo. Racial Characters in Human Dentition, *Proc. Am. Phil. Soc.*, **67**:157, 1928.
2. Jorgensen, K. D. The Dryopithecus Pattern in Recent Danes and Dutchmen, *J. D. Res.*, **34**:195, 1955.

Physical Properties of the Zinc Oxide–Eugenol Impression Pastes

G. E. MYERS and F. A. PEYTON

University of Michigan, School of Dentistry, Ann Arbor, Michigan

The zinc oxide–eugenol impression pastes have been available to the profession since the 1930's and have become increasingly popular in impression procedures in denture prosthesis. Before this time, mixtures of zinc oxide and eugenol were used as dental cements, being mentioned for this purpose in the literature as early as 1887,[1] and such mixtures are still widely used as temporary dental cements. Investigations of the properties of zinc oxide–eugenol mixtures have been directed to an evaluation of either the dental cements or the impression pastes. The basic properties of these cements and impression pastes are, to a large extent, reciprocal. Early investigations[2–7] were largely concerned with desirable compositions of mixtures for impression pastes or dental cements. Later studies[8–10] of the fundamental nature of the reaction between zinc oxide and eugenol have revealed the nature of the setting reaction, and more recently, the clinical and physical properties of commercially available impression pastes have been investigated.[11–14]

A large number of zinc oxide–eugenol pastes are available, which are essentially similar in general properties but differ in their clinical behavior. Variations are noted from one product to another chiefly in relation to setting times, consistency of the mixed material, and the physical qualities of the set impressions, including hardness, brittleness, tackiness, and ease of separation from the case. Products vary also in their reaction to environmental changes and the effects which temperature and humidity have on the setting qualities of the paste. Variations may exist in the effect of storage on the physical and clinical properties of these products. The data here presented represent part of a study of clinical and physical properties of the zinc oxide–eugenol impression materials, which was undertaken in order to compare and evaluate the more commonly used products available to the dentist.

MATERIALS AND METHODS

The fifteen products discussed in this paper were purchased on the open market and are listed alphabetically by their proprietary names in Table 1. All tests were carried out on samples made from materials having the same batch number.

The manufacturer's directions regarding proportioning and mixing were followed for all products. Preliminary tests showed that those pastes supplied in a tube could

This report is compiled from data obtained in studies supported by contract No. DA-49-193-MD-2001, between the Office of the Surgeon General, Department of the Army, and the University of Michigan.

Received for publication January 28, 1960; revised by authors July 27, 1960.

be dispensed by length with sufficient accuracy to give reproducible results if care was exercised. Measured lengths of base and accelerator, which varied with the different test procedures according to the size of sample required, were dispensed onto the pad directly from the tube and the mixing timed accurately.

The progress of the hardening process which occurs as the zinc oxide–eugenol paste sets can be examined by means of a suitable penetrometer. In this study, the Krebs penetrometer with a cylindrical indenter 1.59 ± 0.13 mm. in diameter and with a total load of 50 gm. was used. The freshly mixed impression paste was placed in a metal ring 1 inch in diameter and $\frac{1}{8}$ inch deep, and the top surface of the paste was leveled off. The indenter was applied to the surface for 10 seconds at 30-second intervals, and the penetration noted on the dial gauge. An average of three sets of data at each

TABLE 1

PRODUCTS TESTED

	Product	Batch No.	Date of Purchase	Manufacturer
A	Zorite (slow)	None	June, 1959	H. J. Bosworth Co., Chicago
B	Zorite (fast)	None	June, 1959	H. J. Bosworth Co., Chicago
C	Plastopaste	991	June, 1959	H. J. Bosworth Co., Chicago
D	Coe-Flo	LL14	Jan., 1959	Coe Laboratories Inc., Chicago
E	All-Tec	X2	July, 1959	Coe Laboratories, Inc., Chicago
F	Coe-Trans	1091	Jan., 1959	Coe Laboratories, Inc., Chicago
G	Truplastic	359E	June, 1959	William Getz Corp., Chicago
H	Opotow Impression Paste, Lower	301162	Feb., 1959	Interstate Dental Co., New York
I	Opotow Impression Paste	958496	June, 1959	Interstate Dental Co., New York
J	Luralite	RR955, RR962, 8112	Jan., 1959	Kerr Manufacturing Co., Detroit
K	Impression Paste	SS507, SS5646931	Jan., 1959	Kerr Manufacturing Co., Detroit
L	Krex	459	June, 1959	Lee Smith, Chicago
M	Multiform	00599	Jan., 1959	Surgident Ltd., Los Angeles
N	Hardset	86158302	Jan., 1959	S. S. White Mfg. Co., Philadelphia
O	Impression Paste	7185879	Jan., 1959	S. S. White Mfg. Co., Philadelphia

time interval was taken and the penetration plotted against time to give a curve. During the period when the paste is fluid, the indenter penetrates the specimen completely. As hardening occurs, however, the extent of penetration decreases rapidly until a constant minimum penetration is obtained. The value of the constant minimum reading will vary according to the hardness of the set impression.

For the routine determination of initial and final set time, simpler methods involving less expensive apparatus than the Krebs penetrometer can be used. If a suitably shaped instrument such as a short piece of steel rod of about the diameter of a dental bur shaft is applied to the surface of a freshly mixed zinc oxide–eugenol paste and then removed, the paste will adhere to the rod and string out as it is withdrawn. Repetition of this procedure at definite time intervals will reveal a point at which the paste no longer develops strings when the rod is withdrawn. This change in property occurs at the time of initial set and the loss of the normal working qualities of the paste. In the tests conducted in this study, a steel rod 2.4 mm. in diameter with a flat end and rounded edges and weighing 10 gm. was applied to the surface of the paste at 20-second intervals. The end of the rod was wiped clean after each

application. Initial set time was recorded as the time at which stringing no longer occurred and was recorded to the nearest half-minute. The average for three determinations was taken. Final set was determined with a 1/12-inch Gillmore needle weighing ¼ lb. Freshly mixed paste was loaded in a ring 1 inch in diameter and ⅛ inch deep, and the surface of the paste leveled off. The needle was applied to the surface for 10 seconds at 30-second intervals. At first, the needle penetrated the specimen completely, but, as the paste hardened, the penetration decreased in depth until a series of impressions of approximately equal depth was obtained. The time of the first of such a series of similar indentations was recorded as the final set time.

To compare the initial set times obtained in the rod test described above with the clinical manipulation of these materials, two series of tests were run on the same mixes of eleven zinc oxide pastes. The mix of paste was made according to the manufacturer's directions and divided into two equal parts. One part was used to determine the initial set, using the rod method described above. The initial set of the other part was determined by assessment of the point at which the paste would no longer be used to record an impression. The two sets of times were recorded separately by independent observers and subsequently compared when all eleven materials had been investigated.

The consistency of the zinc oxide paste was determined with the modified slump test, which has been used by other investigators.[11, 12] A measured quantity of mixed paste is placed between two glass plates, and a weight applied for a fixed time. The diameter of the resulting disk of impression material is used as a measure of the consistency of the impression paste. The more fluid the consistency, the larger the disk diameter. In this study, 0.5 ml. of freshly mixed impression paste was placed on a glass plate; 1½ minutes after the beginning of the mix another glass plate weighing 20 gm. was placed on the paste, immediately followed by a 500-gm. weight. Ten minutes after the start of the mix the weight was removed, and the average diameter of the disk was determined to the nearest millimeter by measuring the maximum and minimum diameters. At least three determinations were completed for each product, and the average value recorded. To compare these consistency values with the clinical behavior of the impression pastes, measured quantities of the paste were mixed, and the consistency assessed by watching the flow of the mixed material from the mixing spatula. Three categories can be readily recognized: (*a*) the paste does not flow from the spatula, (*b*) the paste flows slowly from the spatula, and (*c*) the paste flows freely from the spatula.

The hardness of the set impression was measured in this study by the degree of penetration of the penetrometer needle applied to a specimen at ½ hour after the beginning of the mix. A Krebs penetrometer was used with an ASTM D-5 needle, and a total indenter load of 100 gm. The specimen mix was flowed into a metal ring 1 inch in diameter and ⅛ inch in depth. Thirty minutes after the beginning of the mix, the needle was applied to the surface of the specimen and allowed to penetrate for 10 seconds. The extent of penetration was measured to the nearest 0.1 mm. Three such determinations were completed for each of the seven products examined, and the averages, adjusted to the nearest 0.1 mm., were recorded.

Further to examine the properties of the set impression paste, a flow test was per-

formed on cylindrical samples made from standard mixes of eight products. A sample made by filling a mold 10.0 mm. (0.394 inch) in diameter and 6 mm. (0.236 inch) high was subjected to a load of 1 kg. for 10 minutes, and the extent to which the sample shortened was measured. The percentage of shortening was then calculated and recorded as the percentage of flow.

The facility with which an impression can be removed from a cast was examined by filling a metal ring 16 mm. in diameter and 6 mm. high with freshly mixed paste and allowing it to set. The ring was boxed with a strip of wax. Five minutes after final set, a mix of dental stone was poured into the mold so formed. The mold was vibrated to remove air bubbles, and the stone was allowed to harden. Two hours later the mold was immersed in water at 140° F. for 15 minutes, then the cast was removed from the ring of zinc oxide. At least three specimens were prepared for each product, and the surface of each specimen was examined under low-power magnification for the surface quality and the presence of any adherent impression material. The zinc oxide surface was also examined for any indication of adherent stone.

To examine separation of the cast from the impression material under conditions more closely resembling clinical conditions, where the presence of rugae and other surface irregularities of the mucous membrane exist, impressions were taken of a rubber model of an upper edentulous mouth. One impression was taken with each of the fourteen products, using an acrylic tray adapted to the model. The same technique of pouring stone and separating from the impression as described above was followed, and the casts obtained were examined under low-power magnification.

A silicone rubber test block was used to evaluate the ability of the zinc oxide–eugenol products to register fine detail. The rubber test block, approximately 1¼ inch square, was made by duplicating the surface of a metal block on which V-shaped grooves had been cut, having the following widths: 0.0015, 0.0021, 0.0030, 0.0042, 0.0060, 0.084, and 0.0120 inch. A metal ring 1 inch in diameter and ⅛ inch high was placed over the rubber test block so as to include portions of all seven lines. The ring was overfilled with zinc oxide paste, and a small glass plate was placed over the ring. A 50-gm. weight was then applied, and the paste allowed to set. Two minutes after the final set, the impression was removed and examined under low-power magnification to check for the reproduction of all seven lines. The impression was then boxed with wax and poured in stone. One hour later the cast and impressions were immersed in water at 140° F. for 15 minutes and then separated. The stone models were examined for reproduction of the impressions.

RESULTS

The setting characteristics of seven products were studied, using the Krebs penetrometer, and three typical curves are shown in Figure 1. Some products, for example, *F*, harden very rapidly, whereas, with other products, such as *B*, the hardening occurs over a longer period of time.

Values obtained by the rod method of testing initial set are given in Table 2, and range from 1.5 to 4.0 minutes, nine of the values being less than 3 minutes.

The final setting times of fifteen products when tested by the ¼ lb. Gillmore needle are listed in Table 3. These values range from 3.0 to 15.0 minutes.

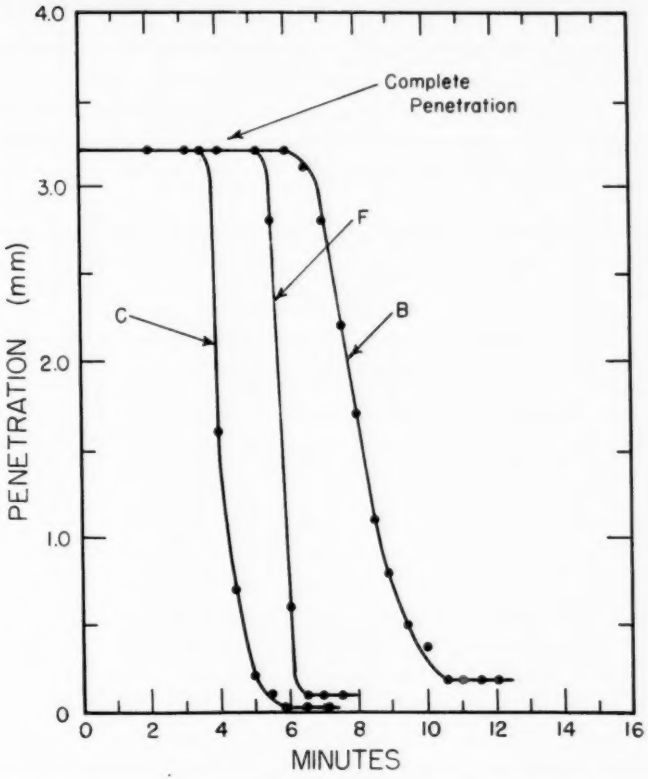


FIG. 1.—Setting characteristics of three zinc oxide pastes as determined by the Krebs penetrometer

TABLE 2

INITIAL SET TIMES DETERMINED BY ROD METHOD AND BY CLINICAL EVALUATION

PRODUCT	INITIAL SET	
	Rod	Clinical
A	3.5	.....
B	2.5	.....
C	2.5	2.5
D	3.5	3.5
E	4.0	.....
F	1.5	1.5
G	3.0	.....
H	3.5	3.5
I	2.5	2.5
J	2.5	2.5
K	3.0	3.0
L	2.5	2.5
M	1.5	1.5
N	2.0	2.0
O	1.5	2.0

The results obtained in the comparison of initial set times as determined by the rod method with those assessed from the clinical behavior of the pastes are listed in Table 2. It can be seen that the values agree for ten of the eleven products.

The consistency values obtained for fifteen products using the modified slump test are shown in Table 4, along with the values recorded in the clinical evaluation of consistency.

The results of the test for hardness of the set impression are listed in Table 5 and range from 0.5 to 3.2 mm.

TABLE 3

FINAL SET TIMES OBTAINED WITH KREBS PENETROMETER AND $\frac{1}{4}$-LB. GILLMORE NEEDLE

PRODUCT	FINAL SET (MINUTES)	
	Gillmore Temp. 76° F. R.H. 59 Per Cent	Krebs Temp. 75–77° F. R.H. 46–49 Per Cent
A	4.0	.....
B	3.0	.....
C	4.0	.....
D	7.0	6.5
E	7.0	.....
F	15.0	.....
G	3.5	.....
H	5.0	.....
I	6.5	6.5
J	5.5	5.5
K	5.0	5.0
L	4.5	.....
M	3.5	3.0
N	5.5	5.5
O	11.5	12.5

The percentage flow of a cylindrical specimen was determined for eight products. The samples were tested at three time intervals after the final set, namely, 5 minutes, 1 hour, and 24 hours. The values obtained are shown in Table 6.

The fourteen products examined for the separation of the impression from the cast were all satisfactory in this respect. With eight of the products, a slight deposit was noticed on the stone when examined carefully under low-power magnification. However, in no case was the deposit of a nature or extent that would be significant in the practical use of these impression materials. In the test series in which impressions were taken of a rubber model of an edentulous maxilla, these results were confirmed, and no product presented any practical problems in respect to separation from the stone cast or quality of the cast surface.

All fourteen products tested registered the finest lines satisfactorily, and all the lines were reproduced in the stone casts. In three instances the impression failed to register the finest line on the first attempt. On repeating the test, however, with

TABLE 4

COMPARISON OF CONSISTENCY TEST VALUES WITH CLINICAL FLOW

(Products Arranged in Order of Their Disk Size)

Product	Disk Diameter (Mm.)	Clinical Flow	Initial Set (Minutes)
J	50	Freely	4.0
K	49	Freely	5.0
A	49	Freely	5.0
B	45	Freely	2.5
D	44	Freely	4.5
C	43	Freely	4.0
E	43	Freely	4.0
G	41	Freely	3.0
M	40	Freely	2.0
N	37	Slowly	3.0
O	37	No flow	4.5
L	34	No flow	3.0
H	34	No flow	3.0
I	32	No flow	2.5
F	27	No flow	1.5

TABLE 5

PENETRATION HARDNESS OF SET IMPRESSION PASTE, KREBS PENETROMETER, 30 MINUTES AFTER MIXING

Product	Penetration (Mm.)	Type
D	0.5	Hard-set
K	0.5	Hard-set
N	0.6	Hard-set
J	1.0	Hard-set
M	1.5	Soft-set
O	1.9	Soft-set
F	3.2	Soft-set

TABLE 6

PERCENTAGE FLOW VALUES OF SEVEN ZINC OXIDE–EUGENOL IMPRESSION PASTES AT THREE TIME INTERVALS AFTER FINAL SET

PRODUCT	PERCENTAGE FLOW AT		
	5 Minutes	1 Hour	24 Hours
D	4.0	0.83	0.60
K	5.1	1.8	2.0
N	5.5	2.4	1.2
J	6.8	4.7	2.8
M	8.8	4.0	2.2
O	13.7	5.4	2.4
F	47.3	26.3	14.1

special attention given to overfilling the ring and applying the weight, a satisfactory impression was obtained.

DISCUSSION

Setting times.—Of interest to the clinician is the time available in which to mix the paste, load a tray, and register an impression before the desirable flow qualities of the paste end with the occurrence of initial set. Such a period of time is customarily described as the manipulation or working time. Once the impression has been registered, a fairly rapid and well-defined hardening of the paste is desirable, and this quality is determined in the measurement of final set. From the slopes of the curves seen in Figure 1, it can be noted that the products differ in the rate at which hardening occurs. Some products, for example, *F*, harden rapidly, whereas with other products, such as *B*, the hardening occurs over a longer period of time. The working time or manipulation time of these pastes can be obtained by recording the time at which the indenter no longer completely penetrates the specimen. The final set can be obtained by noting the time at which the first of three constant minimum indentations is obtained. Another method of selecting the final set point from the penetrometer data is to establish an arbitrary penetration value for final set, for example, 0.2 mm., and consider final set to have occurred when the penetrometer reading is less than that amount. A comparison of the final setting time obtained by the Krebs penetrometer with those obtained by the ¼-lb. Gillmore needle is shown in Table 3. For the seven products compared, the values are the average of three determinations expressed to the nearest half-minute. These determinations by two test procedures were not done on the same mix or at the same time, although conditions of proportioning, mixing time, temperature, and humidity were similar. Although the two sets of values do not match exactly, there is a sufficient correlation for the purpose to which these values are applied. For general testing purposes, it seems that either the Krebs penetrometer or the Gillmore needle can be used satisfactorily to determine the final set of these pastes and that the values from the two methods will correlate reasonably well.

Consistency.—In comparing the clinical quality of flow from the spatula and the values obtained from the modified slump test, the following facts are of interest. Those products which in the slump test had a value greater than 37 mm. flowed freely from the spatula, and those products with a value of less than 37 mm. did not flow from the spatula. Two products had a value of 37 mm. in the slump test but exhibited different qualities of flow from the spatula, one flowing slowly from the spatula and the other not at all. This difference in flow qualities between two products with the same value on the slump test is thought to be due to the difference in initial set times of the two products. The initial set times are listed in Table 4, and it can be seen that the product which is more fluid in the clinical evaluation and flows slowly has a shorter initial set time than the product which clinically exhibits no flow. The influence of this difference in initial set times is responsible for two products, with clinically different consistencies, having the same disk size in the modified slump test, because the shorter initial set time reduces the period during which the 500-gm. weight can act effectively on the specimen. This same factor may be influencing the disk sizes obtained with other

products in the series, and the comparative clinical behavior of two products can be predicted only from their disk sizes when the setting times are approximately the same.

Quality of the set impression.—Certain qualities are necessary in the set zinc oxide paste if an impression is to fulfil its purpose in restorative dentistry. Qualities of special interest are the hardness, freedom from friability, freedom from tackiness, dimensional stability, compatibility with materials used to make a cast, and ease of separation from the cast. A satisfactory impression should be hard and firm enough not to bend or distort on removal from the mouth. It should not be brittle or friable in the set state, so that, if necessary, the impression can be returned to the mouth, and also to facilitate the subsequent pouring of the cast. In considering these properties it should be remembered, however, that the material is always well supported by a suitably designed impression tray. Some slight tackiness in the set impression is found in many products. Provided that this is not present to a degree that the impression will not separate easily from the cast, no clinical difficulties arise from this quality. The zinc oxide pastes have been shown[9] to be sufficiently stable dimensionally for the purpose for which they are employed.

The values for the hardness of the set impression range from 0.5 to 3.2 mm. In relation to the hardness of the set impression, many of the products fall into one of two categories. In one group, often referred to as the "soft-set products," the paste is comparatively soft when set and tends to bend rather than fracture when placed under stress. In the other group, referred to as the "hard-set products," the paste is hard and comparatively brittle in the set state, and when stress is applied to a free specimen, it will fracture. These qualities are related to the composition of the paste and are usually accompanied by other properties characteristic of each group. For example, the soft-set products are, in general, buttery in the freshly mixed state and do not flow readily. They usually set slowly, and the point of initial set is not well defined. On the other hand, the hard-set products usually are free flowing and set rapidly with a well-defined point of initial set. All products, however, do not fall clearly into one or the other of the above groups. Of the seven products tested, the four hard-set materials have values of 1.0 mm. or less, and the 3 soft-set products have values of 1.5 mm. or greater.

If the products are arranged in the order of their flow values as determined 5 minutes after final set, the sequence is the same as that seen when they are arranged in the order of their penetration hardness values. The hard-set products have flow values of less than 7 per cent, and the soft-set products have values of greater than 8 per cent.

SUMMARY

Several physical and clinical properties of the commercially available zinc oxide impression pastes were studied. An attempt was made to correlate the values obtained from bench tests for initial set and consistency with the clinical behavior of the pastes in regard to these properties. Correlation of the values obtained in the final set test using the Krebs penetrometer and the Gillmore needle was examined. The qualities of the set impression were investigated in relation to hardness, flow properties, separation from the cast, and detail reproduction. Initial set times of the fifteen products tested varied from 1.5 to 4.0 minutes. Nine of the fifteen products had an initial set value of less than 3 minutes. The final set times of the fifteen products determined with the

Gillmore needle ranged from 3.0 to 15.0 minutes. Comparison of the values obtained in the rod test for initial set with the clinical behavior of these pastes showed a close degree of correlation. The initial set times as determined represented the time available in which the paste has to be mixed, loaded into a tray, and the impression registered. A comparison of the final set times determined with the Krebs penetrometer and the Gillmore needle shows a correlation sufficiently close for the purpose to which the values are applied. The values obtained in the consistency test varied from 27 to 50 mm. When these values were compared with the clinical behavior of the pastes, it was found that pastes with a disk size in the consistency test greater than 37 mm. flowed freely from the spatula and that those with a disk size less than 37 mm. did not flow from the spatula. Initial set time may influence the disk size obtained in the consistency test. A short initial set time may give a smaller disk size than the consistency merits, as compared with a product of similar consistency but having a longer setting time. For this reason, disk sizes in the consistency test cannot be used as a basis of comparing the relative consistency of two pastes unless they have similar initial set times. From the clinical point of view, the zinc oxide impression pastes can be divided into three groups according to the nature of the flow from the spatula: (*a*) flows freely, (*b*) flows slowly, and (*c*) does not flow. As measured with the Krebs penetrometer, the hardness of the set impression paste in seven products varied from 0.5 to 3.2 mm. Those products described as hard set had values of 1.0 mm. or less, whereas those known as soft-set products had a value of 1.5 mm. or more. The percentage flow of the set impression paste measured 5 minutes after final set for eight products ranged from 3.8 to 47.3 per cent. The hard-set products had values of 7 per cent or less, and the soft-set products had values of 8 per cent or greater. At the end of 1 hour the percentage flow values were considerably reduced, and after 24 hours a further hardening was noted. All fourteen products tested separated satisfactorily from the stone cast. All fourteen products reproduced the finest line on the test impression block satisfactorily, and when the impressions were poured in stone, the lines were all reproduced on the cast.

REFERENCES

1. Tomes, J., and Tomes, C. *A System of Dental Surgery*. London: Churchill, 1887.
2. Ross, R. A. Zinc Oxide Impression Pastes, *J.A.D.A.*, **21**:2029, 1934.
3. Kelly, E. B. United States Patent No. 2,077,48, April 21, 1937.
4. Wallace, D. A., and Hansen, H. L. Zinc Oxide–Eugenol Cements, *J.A.D.A.*, **26**:1536, 1939.
5. Paffenbarger, G. C., and Caul, H. J. *Dental Cements: Dental Centenary Celebration Proc., March, 1940*, p. 232. Baltimore: Waverly Press, 1940.
6. Lawrence, G. O. Impression Pastes, *Austral. D. J.*, **45**:207.
7. Molnar, E. J., and Skinner, E. W. A Study of Zinc Oxide–Rosin Cements, *J.A.D.A.*, **29**:744, 1942.
8. Harvey, W., and Petch, N. J. Acceleration of the Setting of Zinc Oxide Cements, *Brit. D. J.*, **80**:1 and 35, 1946.
9. Copeland, H. I., Brauer, G. M., Sweeney, W. T., and Forziati, A. F. Setting Reaction of Zinc Oxide and Eugenol, *J. Research Natl. Bur. Standards*, **55**:133, 1955.
10. Smith, D. C. The Setting of Zinc Oxide–Eugenol Mixtures, *Brit. D. J.*, **105**:313, 1958.
11. Skinner, W. E., Cooper, E. N., and Ziehm, H. W. Some Physical Properties of Zinc Oxide Eugenol Impression Pastes, *J.A.D.A.*, **41**:449, 1950.
12. Asgarzadeh, K., and Peyton, F. A. Physical Properties of Corrective Impression Pastes, *J. Pros. Den.*, **4**:555, 1956.
13. Clark, R. J., and Phillips, R. W. Flow Studies of Certain Dental Impression Materials, *J. Pros. Den.*, **7**:249, 1957.
14. Vieira, D. F. Factors Affecting the Setting of Zinc Oxide–Eugenol Impression Pastes, *J. Pros. Den.*, **9**:70, 1959.

Electron Microscopy of Enamel and Dentin of Teeth of the Odontaspis *(Selachii)*

WILSON DA SILVA SASSO and HELENA DE SOUZA SANTOS

Departamento de Histologia, Faculdade de Farmácia e Odontologia, University of São Paulo, and Secção de Microscopia Electrônica, Escola Politécnica, University of São Paulo, São Paulo, Brazil

The electron microscope in a very useful tool for the study of dental tissues in normal and pathologic conditions.[1, 2] The majority of studies on tooth ultrastructure deal with human teeth, with few exceptions.[3–6] Information on the ultrastructure of inferior vertebrate teeth is poor, in spite of interest expressed in the subject from the histogenetic point of view.[7]

The nature and structure of fish tooth enamel present many problems and some points as yet not well understood. Some authors[8, 9] consider fish enamel to be a true enamel, but others believe it to be a modified form of dentin which they call "vitrodentin"[10] or "fibrodentin."[11]

The authors[12] have begun a systematic study with the electron microscope of the ultrastructure of the teeth of the shark *Odontaspis*, a shark common to Brazilian coasts.

The aim of the present paper is to give a description of the ultrastructural morphology of the enamel and dentin of the fish *Odontaspis*. Although some doubt exists as to whether the substance which covers the dentin of the shark teeth is true enamel, in this paper, for facility, we shall call it enamel. This, we feel, is justifiable by the observations described in the present paper.

MATERIALS AND METHODS

The replica technique was developed early in electron microscopy for the study of dense materials which could not be obtained in sufficiently thin form for direct observation; it still offers the only means by which fully calcified tissues can be studied *in situ* or in vivo.

Replica studies have been made of the internal structure of enamel and dentin of shark teeth as they exist in surfaces exposed by sawing thick transvers and longitudinal slices; furthermore, replicas of the enamel were also made from the external surface of the tooth. The surfaces were then polished with rouge, and structural details on surfaces to be replicated were enhanced by acid etching for 1–5 seconds in 0.1 N HCl. Positive replicas for electron-microscopic study, as described by Austin and Schwartz,[13] were used throughout this work. The procedure was as follows: a full-strength solution* was applied over the specimen surface and dried before a heat lamp. Excessive

This work was supported, in part, by a grant from the Rockefeller Foundation.

Received for publication March 2, 1960; revised by authors July 14, 1960.

* Zapon Acquanite "A" lacquer, made by Atlas Powder Company.

drying is undesirable. Properly dried, the mechanically stripped film is tough and flexible, but not likely to distort. The positive replica was cast from 0.15 per cent polyvinyl formal in ethylene dichloride. The composite film was placed, the polyvinyl formal side down, on a specimen screen and this upon a coarse wire mesh in a Petri dish. Amyl acetate, as a solvent for the first plastic was added until it just wet the support screen. When the replica was free of the first plastic, usually in about an hour, the support screen and the polyvinyl formal replica could be removed. Image contrast was enhanced by shadow-casting done by the oblique evaporation of a thin coating of metal only a few atoms thick onto the surfaces of the specimen. The replicas were examined in an electron microscope,* and micrographs were taken at a magnification of 1,000–4,000× and examined at magnifications of about 20,000×.

The electron micrographs are direct prints from the positive replicas of the surfaces; thus they represent the actual surface relief.

RESULTS AND DISCUSSION

Figures 1 and 2 are photomicrographs, respectively, of a longitudinal and a cross-section through a shark tooth, to show the relationships between the enamel and dentin and their aspect in the light microscope.

Examination of positive replicas from transvers and longitudinal sections, as well as those from the external surface of the enamel, of the shark tooth, revealed the following morphologic structures in the enamel: a crystal-like component (*CC*); formation resembling the human enamel prisms (*PP*), which we will call "pseudo-prisms"; and a kind of fibrillar substance around the former formation (*FS*).

The crystal-like component is shown in Figure 3 and at the left of Figure 5. In both figures it appears not as a true replica but as material which has been pulled out from the surface of the enamel with the plastic film. It is composed by bundles of thin rods ranging from 200 to 900 A in diameter, arranged parallel to their length. In Figure 3, which is a replica of a longitudinal section of the teeth, these bundles are parallel to the surface of the replica, indicating that the bundles are arranged more or less parallel to the surface of the section and that therefore they are in a plane perpendicular to the surface of the tooth. This observation suggests that the rod axes are probably perpendicular to the external surface of the teeth. In Figure 5 the crystal-like component presented is from a replica taken from the external surface of the enamel. It is worthwhile to mention that the arrangement of the crystal-like component in relation to the axes of the tooth makes the pulling-out detachment much easier in the longitudinal and transverse sections than in the external ones. Thus the crystal-like component shown in Figure 5 is presented only as short rods with a disordered arrangement.

The crystal-like component of the shark tooth is morphologically similar to that of human enamel, as shown by Scott.[1, 14]

The ease with which this crystal-like component is detached from the shark tooth surface makes it a good material for the study of its organization in the enamel, as well as a suitable source for electron diffraction studies.[15] Although there still exist serious doubts concerning the orientation of these crystal-like components in human enamel, in the case of the shark more conclusive evidence could be obtained.

A characteristic electron diffraction pattern of a crystalline substance obtained from

* RCA EMU type.

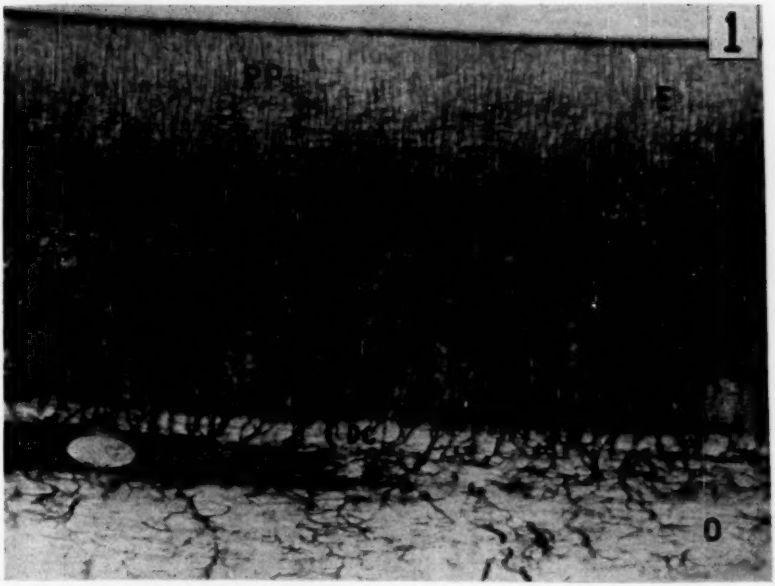


FIG. 1.—Photomicrograph of the shark tooth showing the relationship between enamel (*E*) and dentin (*D* and *O*) and their aspect in the light microscope. *D* = dentin; *O* = osteodentin, *DC* = dentin canals. (Longitudinal section.) (×500.)

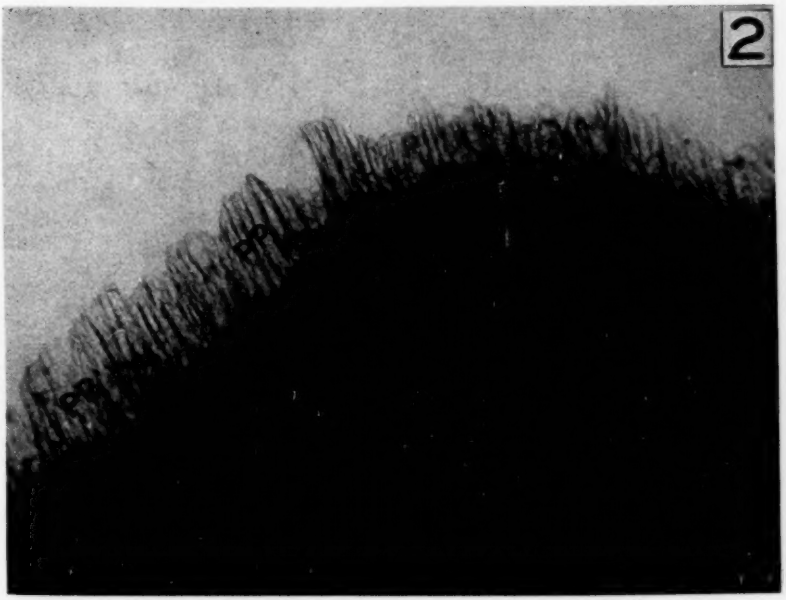


FIG. 2.—Photomicrograph of the shark tooth showing the aspect of the prismlike configurations (*PP*) in the light microscope. (Cross-section.) (×500.)

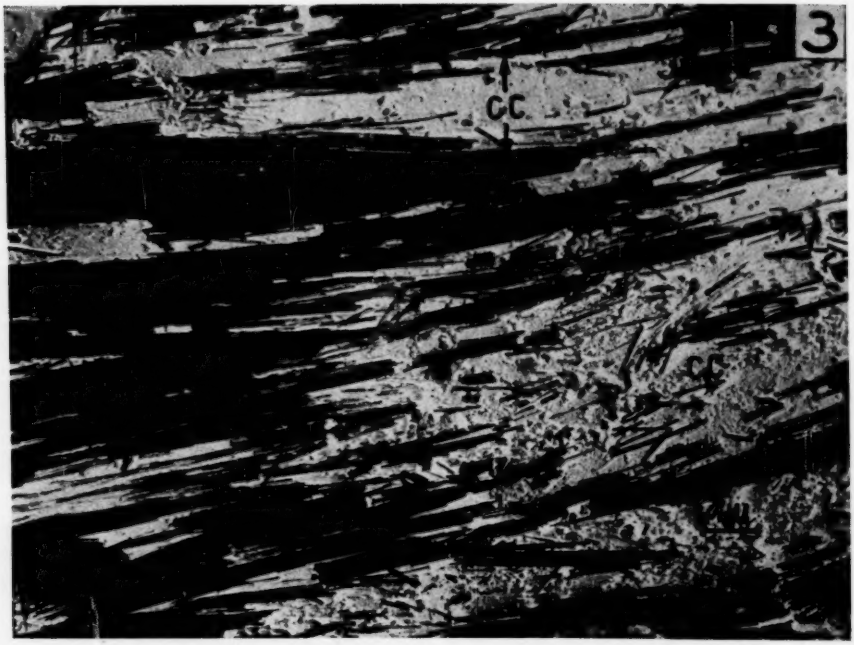


FIG. 3.—Positive replica of shark enamel showing crystalline component (*CC*) as parallel bundles of dark needles. (Longitudinal section.) (×7,000.)

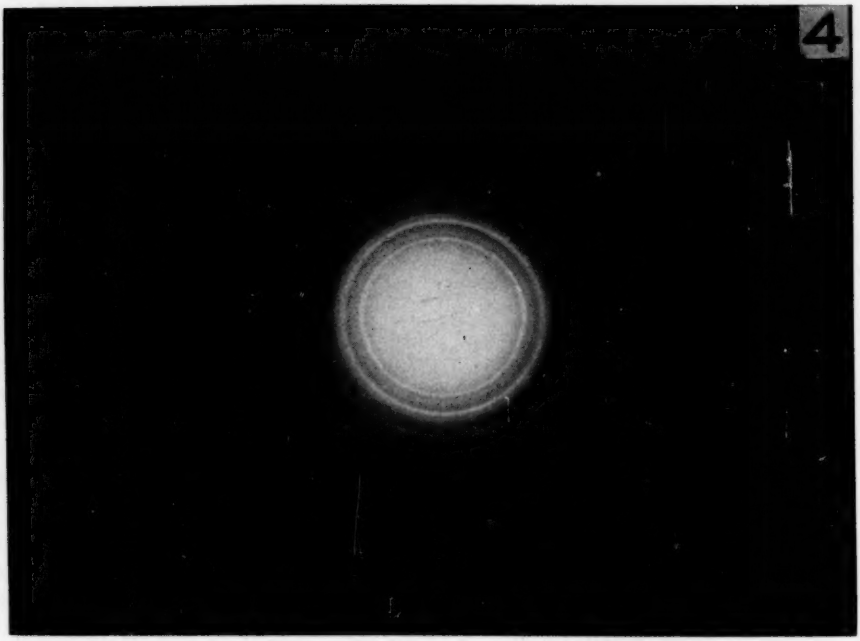


FIG. 4.—Typical electron diffraction pattern of a pseudo-replica of shark enamel showing the presence of apatite crystals.

a pseudo-replica of the crystal-like component is shown in Fig. 4. Measurements of the spacings and relative intensities from this electron diffraction pattern compared with data of X-ray diffraction patterns of natural apatite have shown satisfactory agreement both in the spacing values and in the relative intensities; therefore, it was concluded[15] that the crystal-like component of the shark enamel is crystalline and has the same structure as that of the mineral apatite.

The other components are all shown in Figure 5, which is a replica taken from the external etched surface of the shark tooth enamel. The "pseudo-prisms" (*PP*) appear as depressions with round outlines with thick walls ranging from 0.3 to 1.0 μ; they pre-

FIG. 5.—Positive replica of shark enamel showing the prismlike configurations crosswise (*PP*), crystalline component (*CC*), and fibrillar substance (*FS*). (External surface) (×15,000.)

sent a variable diameter and an irregular arrangement at the tooth surface. Their vicinal positions in relation to the crystal-like component (*CC*) region are also shown in this figure. The fibrillar component appears as an interprismatic substance, as can be seen in Figure 5. They do not present a preferred orientation because fibers of different lengths lying between the prisms can be observed. Their nature is not yet established. Figure 6 is an electron micrograph of the pseudo-prism arrangement taken lengthwise.

The longitudinal view of these pseudo-prisms in true replica, as was observed in human enamel,[1] was found very difficult to obtain because of the ease with which the crystals were detached from longitudinal section positive replicas. This observation could be considered as indirect evidence that the pseudo-prisms of the shark teeth are formed by this crystal-like component.

The examination of positive replicas of shark teeth in transverse and longitudinal

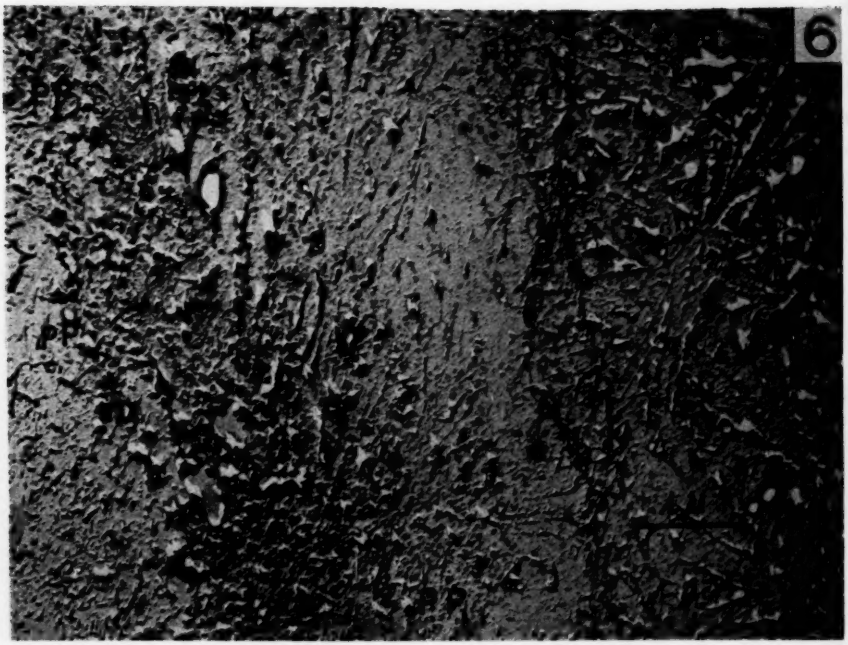


FIG. 6.—Positive replica of shark enamel showing the prismlike configurations lengthwise (*PP*) and true replicas of the crystalline component (*CC*). (Longitudinal section.) (×15,000.)

FIG. 7.—Positive replica of shark dentin showing the dentin canals (*DC*), dentin fibers (*DF*), structureless fibers (*SF*), and matrix (*M*). (Cross-section.) (×1,800.)

sections revealed the following morphological structures in the dentin: dentin canals (*DC*), thin-walled fibers (*DF*), structureless fibers (*SF*), and a homogeneous matrix (*M*) among the former structures. Figure 7 shows the general aspect of the shark dentin in which the dentin canals, thin-walled fibers, matrix, and structureless fibers can be seen.

The canals appear as round depressions with a less dense core, some of them presenting a dark mass which can be considered a dentin fiber. These structures are better shown in Figure 8, in which the fiber inside the canal can be clearly seen as a dark mass placed outside the center. These dark masses are short fragments of the fibers,

FIG. 8.—Positive replica of shark dentin showing the dentin canals (*DC*), matrix (*M*), and isolated dentin fiber (*DF*). (Cross-section.) (×7,000.)

broken during the replica process, while the longer pieces of fibers can be observed in Figures 7 and 8 to lie flat on the surface of the replica. These isolated fibers appear like flattened ribbons having a dark, thicker part which casts a longer shadow than the other part. The canals are distributed irregularly along rows which in positive replicas appear as thicker bands, as can be clearly seen in Figure 7. At a higher magnification (Fig. 8), these thicker bands seem to be constituted of thin structureless fibers (*SF*). The same structureless fibers also appear scattered throughout the dentin matrix but seem to converge from the row regions where the canals lie. The nature of these fibers is not yet understood. The disposition of these canals is different from the arrangement of the human, but both present the dentin fiber inside them.

Collagen fibers presenting their characteristic axial period were not observed in the shark dentin matrix. This absence could be due to the choice of the chemical etching.

The thin-walled fiber somewhat resembles, in a general way, the Tomes fibers of the human teeth.

A certain number of other structures were observed in the shark dentin. Further work is in progress which will elucidate these new structures and the above pictures of the dentin.

SUMMARY

The present study of transverse, longitudinal, and external surface sections made with the aid of positive replicas of the teeth of the shark *Odontaspis* yielded the following results concerning the ultrastructure of shark enamel and dentin:

1. The general morphology of the ultrastructure of shark teeth appeared to be similar to that of human teeth as far as it was observed.

2. The ultrastructure of the substance covering the shark dentin was morphologically similar to human enamel. This substance was composed of a crystalline component identified as natural apatite[15] and prismlike configurations, whose general arrangement and shape were similar to those of the human. These observations were offered as evidence in support of the belief that the external revestment of the shark teeth is a true enamel.

3. The shark dentin was seen to be composed of canals, thin-walled fibers, structureless fibers, and a matrix. The disposition of the canals was irregular in comparison with human dentin canals. The shark dentin canal, as in the human, presented in its interior a fiber which is believed to be the dentin fiber. Throughout the dentin matrix, structureless fibers were observed which concentrated in regions where the rows of the canals are disposed.

REFERENCES

1. Scott, D. B. Recent Contributions in Dental Histology by Use of the Electron Microscope, *Internat. D. J.*, **4**:64, 1954.
2. Souza Santos, P., and Souza Santos, H. Microscopia electrônica técnicas e aplicações em odontologia, *Rev. assoc. paulista cir. dent. São Paulo,* **11**:13, 1957.
3. Pease, D. C. Electron Microscopy of Sectioned Teeth, *Anat. Rec.*, **110**:539, 1951.
4. Scott, D. B., and Wyckoff, R. W. G. The Study of Enamel by Electron Microscopy, *J.A.D.A.*, **44**:377, 1952.
5. Sognnaes, R. F., Scott, D. B., Ussing, M. J., and Wyckoff, R. W. G. Electron Microscopy of the Enamel of Teeth in Various Stages of Development, *J. D. Res.*, **31**:85, 1952.
6. Takuma, S. The Electron Microscopy of the Enamel Surfaces of Teeth under Various Abnormal Conditions, *J. D. Res.*, **34**:152, 1955.
7. Lison, L. "Les Dents." In *Traité de zoologie,* ed. Pierre P. Grassé, Vol. **12.** Paris: Masson & Cie, 1954.
8. Tomes, C. *Traité d'anatomie dentaire—Humaine et comparée,* p. 88. Paris: Octave Doin, 1880.
9. Widdowson, T. W. *Special or Dental Anatomy and Physiology and Dental Histology,* **2**:67. 7th ed. London: Staples Press, Ltd., 1946.
10. Röse, C. Über die verschiedenen Abänderungen der Hartgeewebe bei niederen Wirtbeltiere, *Anat. Anz.*, **14**:33, 1898.
11. Thomasset, J. J. Recherches sur les tissus dentaires des poissons fossiles, *Arch. anat. histol. embryol.*, **11**:5, 1930.
12. Sasso, W., and Souza Santos, H. Estudo comparado da ultraestrutura do dente humano e do cação (*Odontaspis* sp.) ao microscópio eletrônico, *An. fac. farm. e odontol. univ. São Paulo,* **16**: 197, 1959.

13. AUSTIN, A. E., and SCHWARTZ, C. M. Modification of a Positive Replica Technique for Electron Microscopy, *J. Appl. Phys.*, **22**:847, 1951.
14. SCOTT, D. B. The Electron Microscopy of Enamel and Dentin, *Ann. N.Y. Acad. Sci.*, **60**:575, 1955.
15. SOUZA SANTOS, H., and SASSO, W. S. Electron Diffraction and Electron Microscopy Studies on the Crystalline Component of Enamel of the *Odontaspis* (Selachii), *Experientia*. (In press.)

Hereditary Generalized Microdontia

ARTHUR G. STEINBERG, JOSEPH F. WARREN,† and LEONARD M. WARREN‡*

Western Reserve University, Cleveland, Ohio

Microdontia is a rare phenomenon. According to Boyle,[1] "in general microdontia, the teeth are small, the crowns short, and normal contact areas between the teeth are frequently missing." Shafer, Hine, and Levy[2] divide microdontia into three types: (1) microdontia involving only a single tooth; (2) relative generalized microdontia due to relatively small teeth in large jaws; and (3) true generalized microdontia, in which all the teeth are smaller than normal. According to these authors, aside from its occurrence in some cases of pituitary dwarfism, true generalized microdontia is exceedingly rare. A search of the literature has impressed us with the accuracy of this statement.

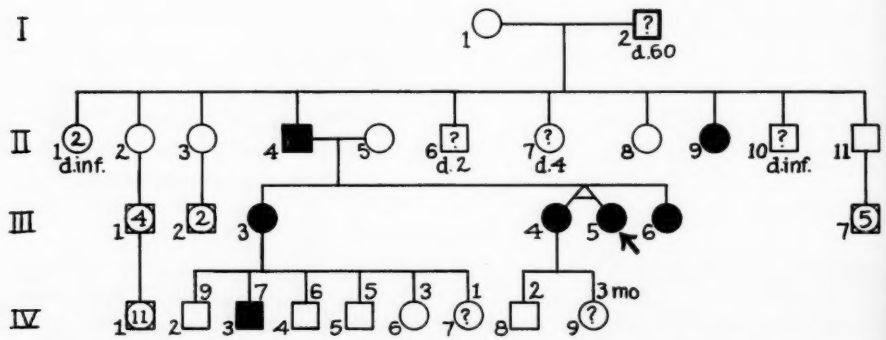


FIG. 1.—Pedigree of family with microdontia. The solid symbols represent those with microdontia. See the text for further details.

We have not found any report of a familial occurrence of true generalized microdontia. It is not mentioned as a hereditary disorder in Witkop's[3] compilation of hereditary oral diseases. This paper is a report of a family in which generalized microdontia has occurred in three generations.

CLINICAL OBSERVATIONS

Three members of the family have been examined in detail, namely, twin sisters aged thirty-two (Fig. 1, *III-4* and *5*) and their sister aged thirty-four (Fig. 1, *III-3*). The general configuration of their faces presents a retrognathic condition. There is a

Received for publication April 8, 1960; revised by authors August 2, 1960.

*Department of Biology.

† School of Dentistry.

‡ Graduate student in orthodontics.

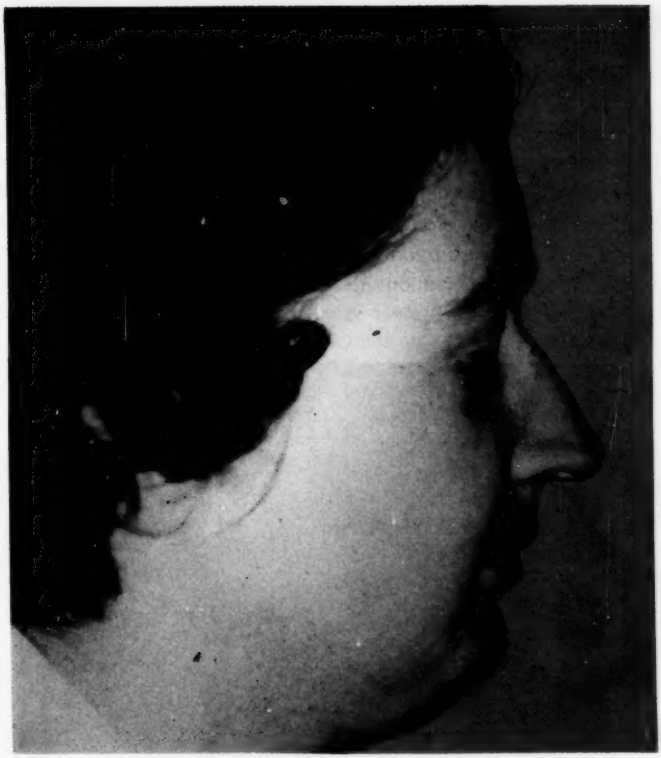


FIG. 2.—Profile view of the proband (Fig. 1, *III-5*)

FIG. 3.—Photograph of teeth of *III-5*, Fig. 1. The upper teeth are capped. Note the interdental spaces in the anterior area.

decided loss of vertical dimension in the lower half of the face, and a closed-bite appearance (Fig. 2). The number of carious lesions present is not significantly more than would be expected of patients in this age group. The condition of the gingivae in general is healthy and of a very tight nature. Interdental spaces are evident in the anterior area but do not exist in the posterior (Figs. 3 and 4). Abrasion is more apparent in the anterior area, where the clinical crowns are reduced to about one-quarter the size of a normal tooth. Both mandibular and maxillary teeth have a discolored, dwarfed appearance, resembling the deciduous dentition in size (Fig. 5). These teeth have a yellowish light-brown hue and appear smaller in the anterior region than in the remainder of the dentition. The dental anatomy of each tooth is similar to its normal counterpart, but in a miniature aspect. X-ray examination indicates that there is a very thin layer of enamel on the crowns of the teeth and that the roots also have dwarf-like characteristics similar to the clinical crowns; however, the bone structure of both alveolar processes appears normal in formation but not in size (Fig. 4).

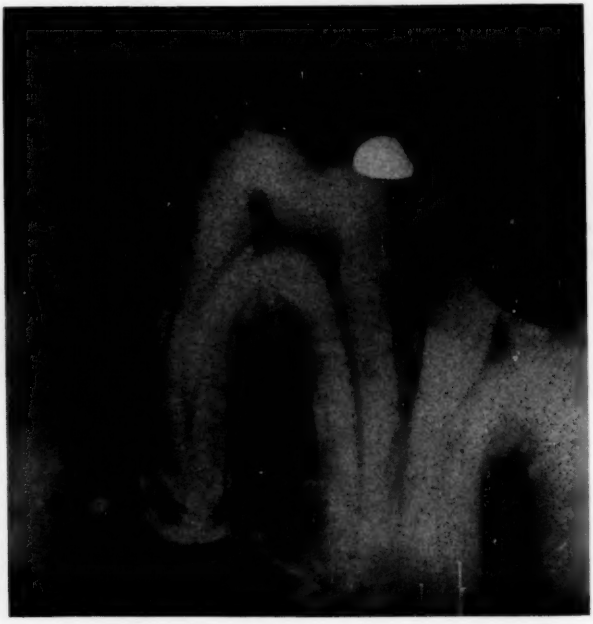


FIG. 4.—X-ray of bicuspid and molar area of *III-5*, Fig. 1

THE PEDIGREE

Figure 1 presents the pedigree as derived from interviews of *II-4* and *5*, *III-3*, *4*, and *5*, and from observation of *III-3* through *III-6* and *IV-2* through *IV-9*. In the figure, affected persons are represented by solid black symbols. Symbols containing question marks represent individuals who were young at the time of death and therefore about whom no information is available (*II-1*, *6*, *7* and *10*) or who are now living but

are too young to be classified (*IV-7* and *9*) or about whom we have conflicting information (*I-2*).

I-2, the grandfather of the proband, was reported by his son (*II-4*) and his daughter-in-law (*II-5*) to have had microdontia. However, his dentist, an alert man in his late seventies, could not recall that this man, who had died 20 years before, had had microdontia. The dentist does recall, however, that *II-4*, who was also his patient, had microdontia.

Evidence from the pedigree suggests that *I-2* did have microdontia, since two of his children had such teeth. If *I-2* did have generalized microdontia, the pattern of transmission is characteristic of dominant autosomal inheritance, with, as far as can be told from this pedigree, essentially complete penetrance. If he did not have microdontia, the condition could be due to a sex-linked dominant gene with failure of penetrance

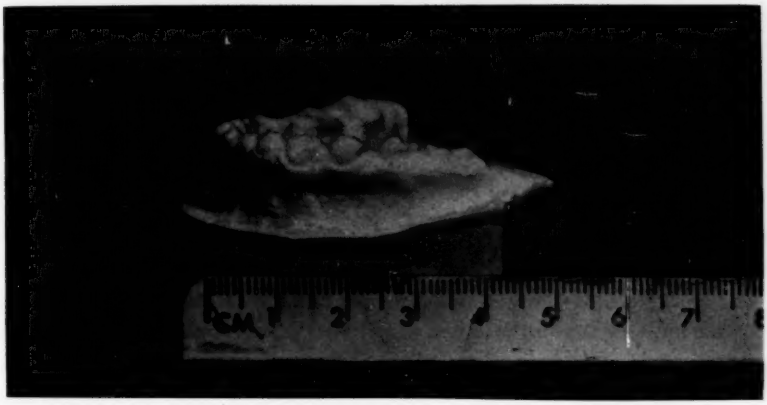


FIG. 5.—Photograph of a cast of the lower teeth of *III-5*, Fig. 1

in *I-1* or to a dominant autosomal gene with failure of penetrance in *I-1* or *I-2*. Since at least two children were affected in generation *II*, it is unlikely that a mutation occurred in *I-1* or *I-2*.

About 50 per cent of the offspring of the affected individuals may be expected to be affected, while if penetrance is complete, none of the descendants of the unaffected individuals will be affected. If penetrance is incomplete, it is possible that the descendants (about 50 per cent) of some of the unaffected individuals will be affected. While we do not have enough data to determine the completeness of penetrance, those we have suggest that it is high.

A point to be emphasized is that two generations of this family had been known to be affected for over 20 years and three generations for 7 years before it was studied genetically. It is probable that other such families exist and, indeed, that families with other genetically determined but unreported dental anomalies exist. They should be studied and reported.

The authors wish to express their gratitude to Dr. William Lawther who took the photographs for Figures 2, 3, and 4.

REFERENCES

1. Boyle, P. E. *Kronfeld's Histopathology of the Teeth,* p. 14. Philadelphia: Lea & Febiger, 1955.
2. Shafer, W. G., Hine, M. K., and Levy, B. M. *A Textbook of Oral Pathology,* p. 26. Philadelphia: W. B. Saunders Co., 1958.
3. Witkop, C. J., Jr. *Genetics and Dentistry.* In Helen G. Hammons (ed.), *Heredity Counseling.* New York: Hoeber, Inc., 1959.

Effect of Microstructure on the Physical Properties of Cobalt-Base Alloys

KAMAL ASGAR and FLOYD A. PEYTON
University of Michigan, School of Dentistry, Ann Arbor, Michigan

Relatively little is known about the microstructure of cobalt-base alloys in dentistry. Manufacturers of dental cobalt-base alloys have published very little on this subject. No research has been conducted on the microstructure of the cobalt-base alloys used in dentistry. The need for research in this field is recognized, however, by both the manufacturers of the alloys and researchers in the dental field.

It appears that it would be profitable to dentistry if a study were made in which the effect of different casting conditions on the grain size and the microstructures of the cast product was established. It would also be useful to determine the physical properties of cast pieces having different microstructures. Today the mechanical properties listed for dental alloys vary a great deal from one type of alloy to another and also within the alloy. For example, elongation values from very small values to as high as 12 per cent are given for these alloys, and the modulus of elasticity determined for these alloys varies from approximately 20 to 40 million pounds per square inch.[1, 2] No satisfactory explanation is given for such a wide range of different physical properties. A study seems to be in order, therefore, to check these values and to determine whether such a variation is inherent in these alloys or is due to different testing conditions, such as sample size, casting condition, or equipment used. With these problems in mind, this study was undertaken in an effort to answer, with a certain amount of confidence, some of the above questions, the importance of which is becoming increasingly more obvious to the dental profession.

MATERIALS AND METHODS

In this study more than 120 specimens were cast under different conditions, and their physical properties as well as their microstructures were studied. All specimens were cast of a cobalt-base alloy* which is of a composition similar to a typical dental alloy.† The investment used was a phosphate-bonded type.‡ Specimen size and manner of sprue ing were patterned after the recommendation of the A.D.A. specification for chromium-cobalt alloys.[3] The conditions studied were related to changing the metal casting tem-

Presented before the Dental Materials Group of San Francisco, California, March, 1959.
This study was supported by a grant from the Office of Naval Research, Contract No. NR-180-360, to the University of Michigan, School of Dentistry.
Submitted by Kamal Asgar to the H. H. Rackham Graduate School of the University of Michigan as part of the thesis required for the Ph.D. degree.
Received for publication April 11, 1960; revised by authors August 18, 1960.

* Stellite 21.
† Vitallium.
‡ Ransom and Randolph No. 711.

perature, varying mold temperature, and heat-treating of the alloys. Melting and casting of the metal were carried out by using an induction casting machine.* The temperature of the molten metal in each melt was checked by means of an optical pyrometer.† The following four conditions were studied:

1. Molten metal was heated to 2,600° F., then cast into a mold of 1,600° F., and then allowed to cool on the bench to room temperature.

2. Molten metal was heated to 2,600° F., cast into a mold of 1,300° F., and bench-cooled.

3. Molten metal was heated to 2,800° F., cast into a mold of 1,600°F., and bench-cooled.

4. As in condition 1, the metal was heated to 2,600° F., cast into the mold of 1,600° F., and bench-cooled. The castings then were heat-treated by placing them in an oven of 1,600° F. for 1 hour and were then quenched in water.

After obtaining the physical properties of the cast specimens, one half of each specimen was imbedded in a plastic-type material for microstructure observations. In this way, the number of samples prepared for microstructure studies was equal to that for physical testing. Study of the microstructure at the fracture point rather than at any other point on the specimen was preferred. By knowing the microstructures at the fracture points, not only their general conditions could be studied, but also the cause of the fracture could be detected.

The results of the physical properties obtained in this study are reported separately.[2]

RESULTS AND DISCUSSION

The plastic material used to imbed the specimens formed a hard mass which did not smear during polishing. Although Stellite 21 is a hard alloy and does not smear during polishing and is not heat-treatable, it may be easily cold-worked. In polishing, special care should be exercised not to cold-work it excessively, and, by etching and polishing two or three times, any cold-worked surface may be removed. After being polished, the specimens were etched. According to Berglund,[4] alloys which have special resistance to attack by chemical reagents, such as high stainless steels and nickel-chrome alloys, are best etched electrolytically. Since Stellite 21 falls in this category, the specimens were etched electrolytically. Different solutions, such as dilute phosphoric acid, 10 per cent solution of oxalic acid, 5 per cent solution of hydrochloric acid, and dilute solution of boric acid, were tried as electrolytes, with results varying from poor to fair. Following the recommendation of Faulkner[5] at Haynes Stellite Company, a 2 per cent solution of chromic acid was used as an electrolyte, and the results were entirely satisfactory. Consequently, all the specimens were etched electrolytically, with low current produced by a 6-volt battery, using a 2 per cent solution of chromic acid as an electrolyte.

Stellite 21 does not have a simple microstructure, but instead it consists of an austenitic matrix, composed of the solid solution of cobalt and chromium as cast, and the matrix has a cored dendritic structure, as shown in Figure 1. According to Badger and Sweeney,[6] the microstructure of Stellite 21 consists of three different types of carbide formations, as well as a eutectic and eutectoid structure similar to pearlite in formation. Three different types of carbide can be detected by using different types of etching solu-

* Williams Inductocast Unit. † Leeds and Northrup.

tions. If a very light electrolytic etch in 2 per cent chromic acid is used, followed by an alkaline permanganate etch of about 7 seconds, the different carbides will produce a selective staining action. One of the carbides, which contains both cobalt and molybdenum in solid solutions, had a brown stain, and the formula Cr_4C was given to it.[6] It is believed that this carbide possesses a cubic structure. Another carbide with a chemical formula of Cr_4C_3 having a hexagonal structure also contains both cobalt and molybdenum in solid solution, and its color varies from very pale yellow to light tan. The third type has a general formula of M_6C, where M may be cobalt, chromium, or molybdenum, and this type of carbide reveals no definite structure. The color of this type of carbide

FIG. 1.—Microstructure of Stellite 21. (Mag. ×500.)

may vary from red to green and occasionally from yellow to blue. In Figures 2 and 3 these three different types of carbides can be seen. The black-and-white photographs, however, obviously do not show the various colors obtained.

According to Grant,[7] however, Badger and Sweeney's identification of the carbides is not complete, and the evidence is insufficient. Grant claims that the austenitic matrix of the alloy consists of perhaps two or three phases of carbides or a carbide of unknown composition.

It is beyond the scope of this study to check the composition of carbides and to accept either Grant's theory or the three different formulas for the carbides suggested by Badger and Sweeney. The purpose of this study, however, is to investigate the effect of the microstructure of a specimen on its physical properties. In general, regardless of the three types of carbides which may exist in any specimen, the microstructure of the alloy

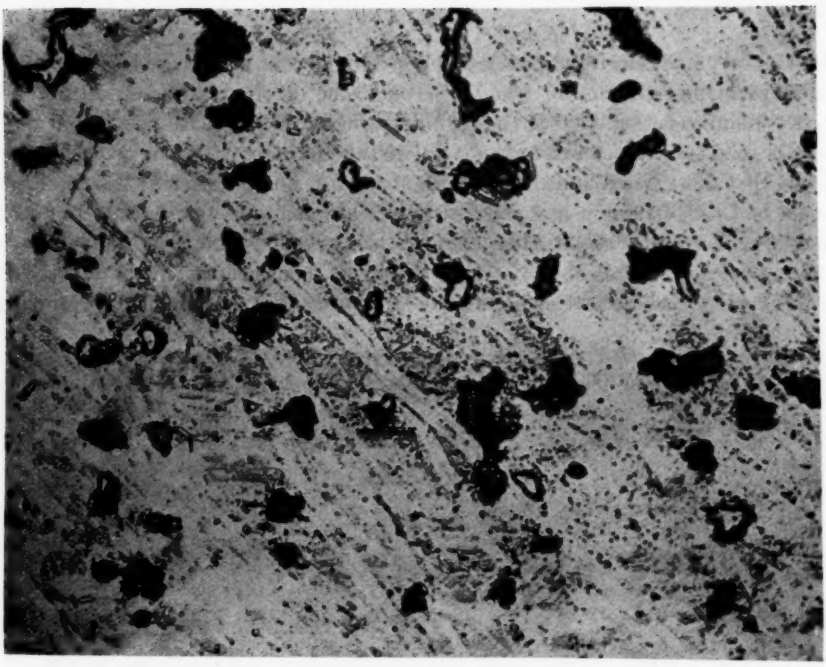


FIG. 2.—Microstructure of Stellite 21. (Mag. ×800.) Different types of carbides

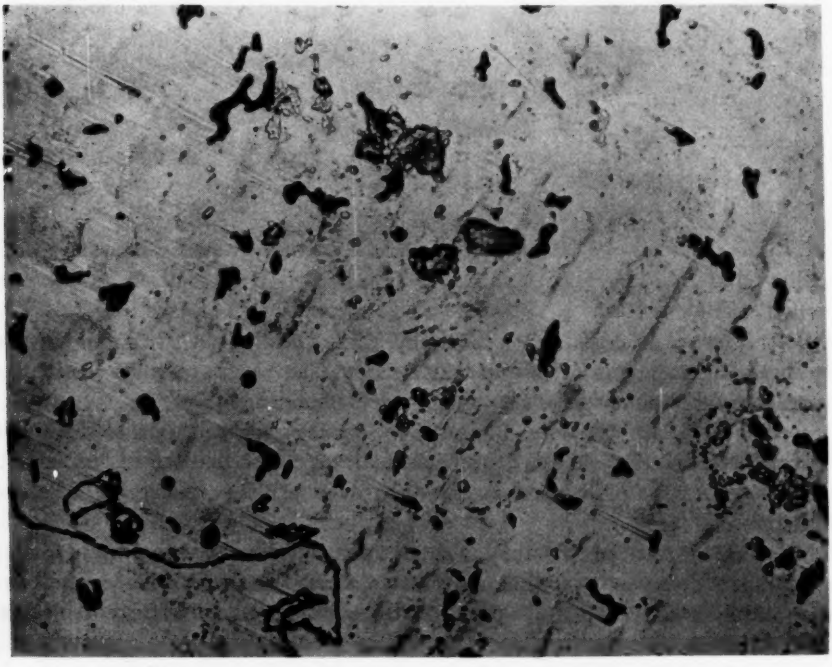


FIG. 3.—Microstructure of Stellite 21. (Mag. ×800.) Different types of carbides

under consideration may represent four distinct types: (1) carbides in the form of grain boundaries and continuous in nature, as shown in Figure 4; (2) carbides that are spherical and discontinuous like islands, as shown in Figure 5; (3) dark eutectoid areas which are present close to and along the grain boundaries, as shown in Figure 6; and (4) a large percentage of these eutectoid areas, which are lamellar in nature, as shown in Figure 7.

It was found that the presence or absence of any one of these four arrangements in the microstructure has a definite effect on the physical properties of the alloys. For example, Figure 8 is a photomicrograph of a representative structure in a series of twenty

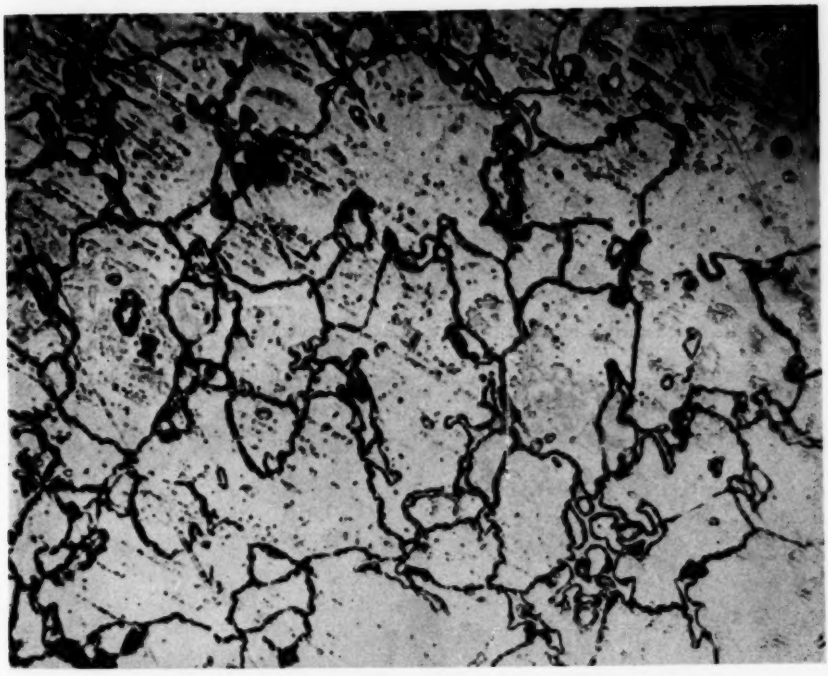


FIG. 4.—Microstructure of Stellite 21. (Mag. ×500.) Continuous carbides

samples. In general, all the microstructures of this series showed many carbides forming within grain boundaries. This type of structure generally showed lower elongation values, and the average elongation of this group, which consisted of twenty cast specimens, was 3.4 per cent. Although all the specimens of this group were cast under similar conditions, for some unknown reason their microstructure was not alike in all specimens, nor did they have similar elongation values. It is interesting to note, however, that if, for any reason, they possessed a different type of microstructure from that in which the carbides formed within the grain boundaries, they also possessed a different value of elongation. For example, four of the twenty specimens of this group had elongations of 6.6, 7.0, 8.0, and 7.2 per cent, which was much higher than the average elongation of only 3.4 per cent for the entire series of twenty samples. They also possessed a type of microstructure different from that representative of this group. In Figure 9 is shown

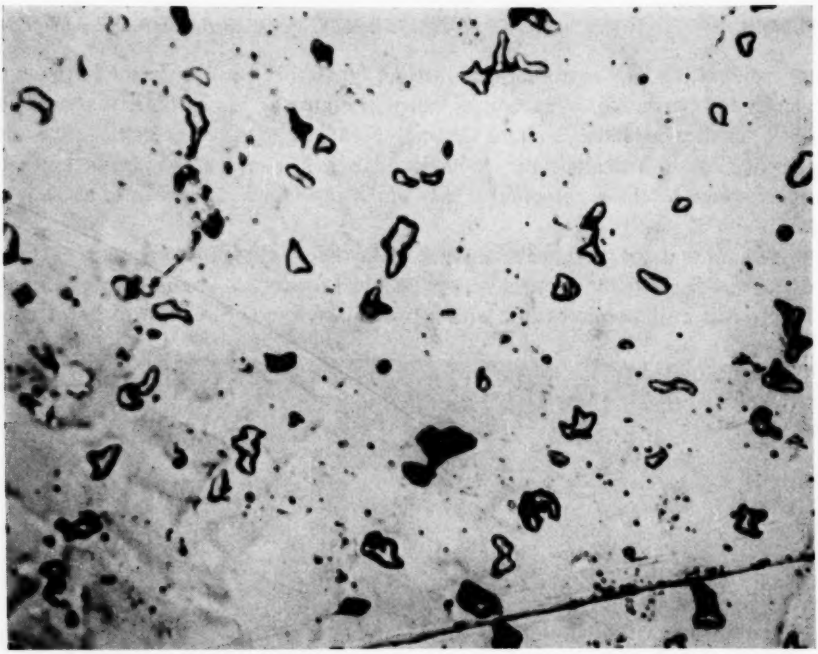


FIG. 5.—Microstructure of Stellite 21. (Mag. ×500.) Spherical and discontinuous carbides

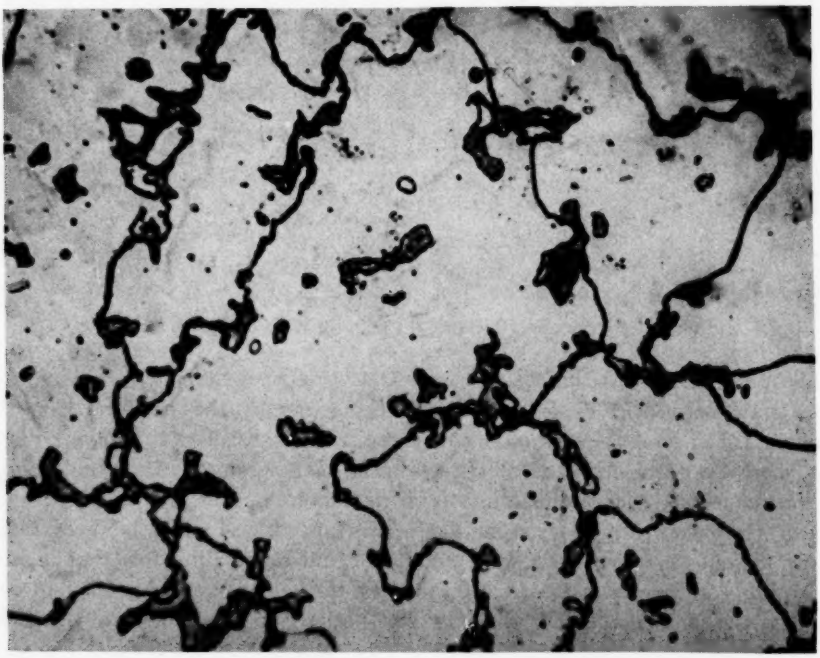


FIG. 6.—Microstructure of Stellite 21. (Mag. ×500.) Dark eutectoid areas present close to grain boundaries.

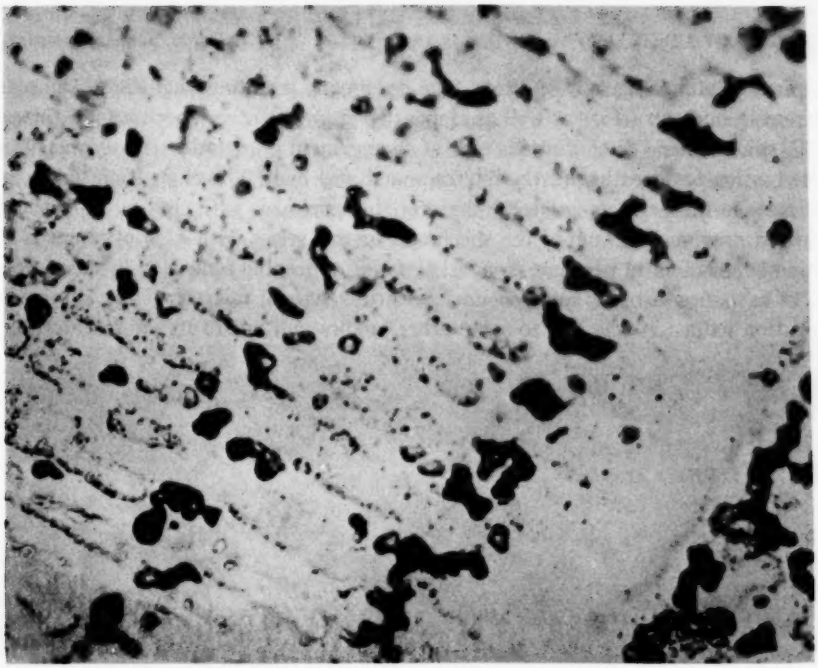


FIG. 7.—Microstructure of Stellite 21. (Mag. ×500.) Larger percentage of entectoid areas lamellar in nature.

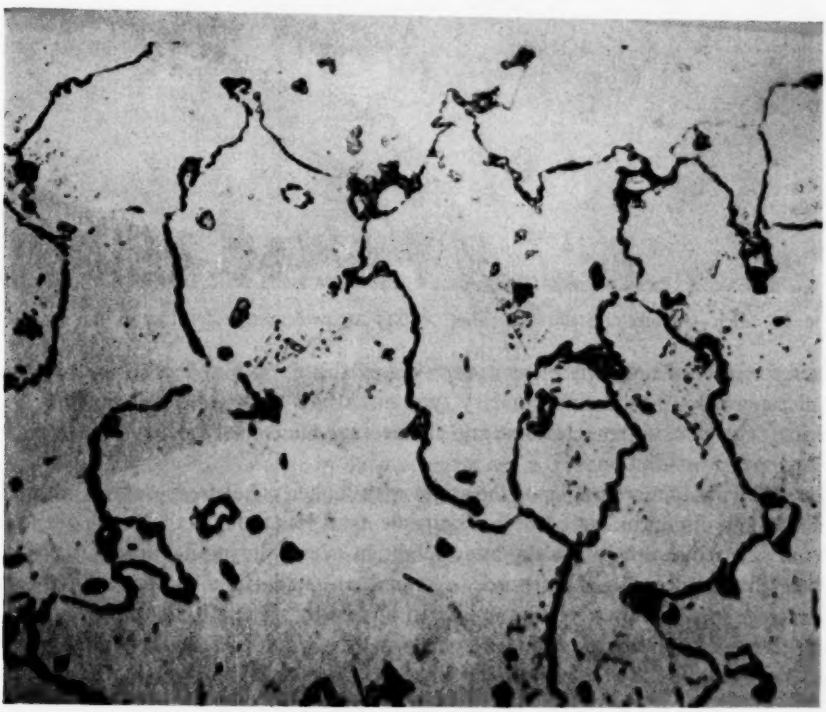


FIG. 8.—Microstructure of Stellite 21. (Mag. ×500.) A typical structure of a series of twenty samples

a typical photomicrograph of these four specimens, showing that these samples are different in microstructure as well as elongation from the other specimens in this series.

The spherical and discontinuous type of arrangement of carbides usually was obtained if the casting temperature of the molten metal was high. The elongation values of the specimens in this series were higher than for the specimens with continuous carbides, as discussed previously. Some of the specimens of this series with discontinuous carbides showed as much as 12 per cent elongation and a noticeable reduction in cross-sectional area of cast specimens. If any specimen with this type of microstructure showed lower elongation values, it was due to some other factors and not to its microstructure. One

FIG. 9.—Microstructure of Stellite 21. (Mag. ×500.) A representative structure of four specimens

reason for some of the lower elongation values of this series may have been the presence of microporosity. The brain boundary type of carbide occurred less frequently in this series of high casting temperatures, and the average number of grains was as low as two or three per cross-section.

Since the photomicrographs of the series with higher metal temperatures showed the spherical and discontinuous carbide arrangements, it was thought that some of the small amount of carbon present in the alloy might burn out during heating of the alloy to higher temperatures. In other words, a somewhat smaller amount of carbides seen in the micrographs of this series is thought to be due to the fact that the specimens cast under this condition may have ended by having less carbon and thus less carbides, which may have caused them to be discontinuous rather than continuous. To check this hypothesis, two specimens, one cast at 2,600° F. and another cast at 2,800° F., were pre-

pared, and total carbon content was determined analytically. The results of this analysis showed that both specimens contained an equal amount, 0.23 per cent, carbon. These results agree with the results of a chemical analysis of the ingots made by the Haynes Stellite Company, in which the carbon content was reported to be 0.25 per cent. From these determinations, it can be observed that the total carbon content remains the same but that the carbide arrangement is different.

The presence of dark areas close to the grain boundaries, which Badger and Sweeney identified as eutectoid areas, may be detected in specimens cast under any one of the

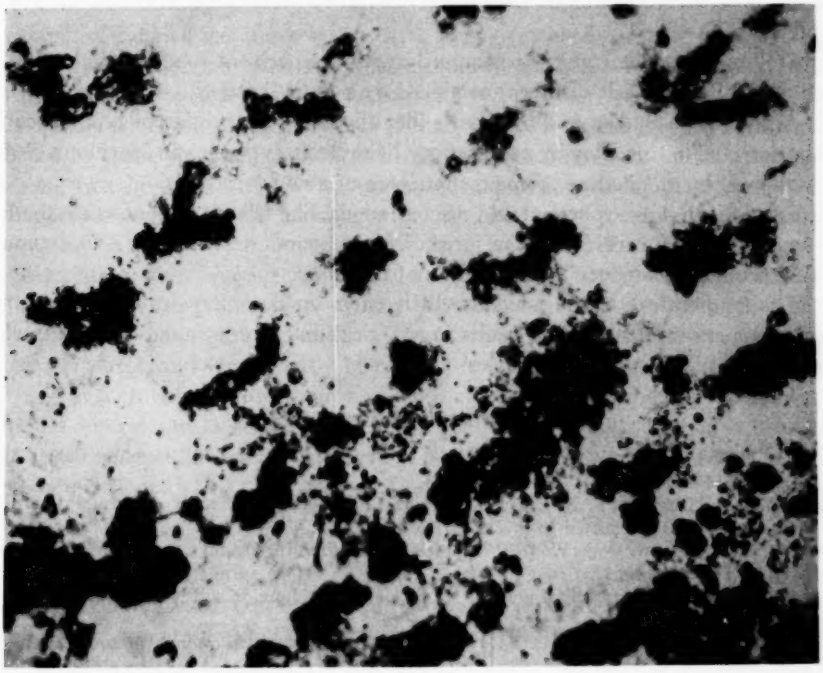


FIG. 10.—Microstructure of Stellite 21. (Mag. ×500.) Specimens were heat-treated; structure shows larger eutectoid areas.

conditions used in this study. This structure appears more frequently in the specimens cast at lower temperatures.

The micrographs of the specimens which were heat-treated showed larger eutectoid areas. These areas were lamellar in nature, as shown in Figure 10. The presence of this type of structure shows lower elongation and yield strength, as is shown in another report.[2]

In this study it was found that, in order for the castings of this alloy to have higher elongation values, they not only should be sound and free from microporosity, but also their microstructure should be of a definite type. Spherical and discontinuous types of carbides which are free from eutectoid composition and microporosities possess greater ductility and elongation. Higher metal casting temperatures reult in such a microstructure. It is important, however, to note that as the metal casting temperature increases,

the interphase reaction between the metal and the investment also increases. It should be remembered that the temperatures used in casting were 2,600°, 2,800°, and 3,000° F. Castings of 2,600° F had the best surface; with 2,800° F., the metal was somewhat darker and less desirable. The surface of the castings of 3,000°F. metal was very poor and practically unacceptable, but the specimens were more ductile, and the possibility of having microporosities was reduced considerably.

SUMMARY

1. Microstructure as well as microporosities have a pronounced effect on the physical properties of Stellite 21.

2. Stellite 21 can be etched satisfactorily with a 2 per cent solution of chromic acid as an electrolyte and a low current produced by a 6-volt battery.

3. According to Badger and Sweeney's identification, three different types of carbide can be detected in this alloy by using a very light electrolytic etch in 2 per cent chromic acid followed by an alkaline permanganate etch of about 7 seconds.

4. Carbides that are spherical and discontinuous, like islands, demonstrate the highest elongation. The carbides in the form of grain boundaries which are continuous or dark eutectoid areas reduce the elongation of this alloy.

5. The temperature of the mold has little effect on the microstructure. High metal casting temperatures, however, results in spherical and discontinuous carbides, which, in turn, yield higher elongation values.

REFERENCES

1. Taylor, D. F. Leibrfita, W. A., and Alder, A. G. Physical Properties of Chromium-Cobalt Alloys, J.A.D.A., **56**:343, 1958.
2. Asgar, K., and Peyton, F. A. Effect of Casting Conditions on Some Mechanical Properties of Cobalt-Base Alloys, submitted to *J. D. Res.*, March, 1960.
3. Taylor, D. F., and Sweeney, W. T. A Proposed Specification for Dental Chromium-Cobalt Casting Alloys, *J.A.D.A.*, **54**:44, 1957.
4. Berglund, T. *Metallographer's Handbook of Etching*, p. 89. London and New York: Sir I. Pitman & Sons, Ltd., 1931.
5. Faulkner, W. H., Haynes Stellite Company, Kokomo, Indiana. Personal communications, November, 1958.
6. Badger, F. S., and Sweeney, W. O. *Metallurgy of High Temperature Alloys Used on Current Gas Turbine Designs: Symposium on Material for Gas Turbine*, p. 99. Philadelphia: American Society for Testing Materials, 1946.
7. Grant, N. J. The Effect of Composition and Structural Changes on the Rupture Properties of Certain Heat Resistant Alloys at 1500° F., *Trans. Am. Soc. Metals*, **39**:368, 1947.

Effect of Casting Conditions on Some Mechanical Properties of Cobalt-Base Alloys

KAMAL ASGAR and FLOYD A. PEYTON
University of Michigan, School of Dentistry, Ann Arbor, Michigan

The casting of cobalt-base alloys in dentistry is comparatively new in relation to the casting of gold alloys and other restorative procedures. It has been less than thirty years since these alloys were introduced to dentistry, but each year they have become more popular. A survey of the choice of dental alloys for removable partial dentures conducted by Lane[1] in 1949 showed that five times as many removable partial dentures were cast of the cobalt-base alloys as of gold alloys. Today this ratio may be even higher, since cobalt-base alloys are the choice of many dentists and laboratory technicians.

Unfortunately, even with such a high demand for this type of alloy in dentistry, little is known by the profession about the physical and mechanical properties of the alloy or about its basic nature. Similar alloys of cobalt are widely used in industry at high temperatures, and consequently more work is done and reported with this type of alloy used at high temperatures than at room temperature. Although acceptable dental techniques for casting these alloys are available, a completely dependable, accurate, and easy-to-apply technique has not yet been developed. Such a technique probably cannot be developed until more is known about the basic nature of these alloys and about the effect of variations in casting procedures upon their properties.

The purpose of this study was to determine the physical properties of cobalt-base alloys and to relate these properties to the microstructure, as well as to observe the influence of casting variables on both microstructure and physical properties. In this way the most acceptable casting practice can be selected which is revealed by both physical tests and microstructure.

MATERIALS AND METHODS

Preparation of specimens.—The size of the specimens in this study was patterned after a previous study conducted by Bush, Ingersoll, and Peyton at the University of Michigan School of Dentistry.[2] The A.D.A. Specification No. 14 for the dental chromium-cobalt alloys[3] also employs the same dimensions for the tensile test samples.

The test bar was $1\frac{3}{8}$ inches in length, 0.09 ± 0.01 inch in diameter, and threaded at the ends with 12–24 threads. The size and shape of the test bars are shown in Figure 1. For the sake of convenience, the dimensions of the test bars were machined into a

This study was supported by a grant from the Office of Naval Research, Contract No. NR-180-360, to the University of Michigan, School of Dentistry.

This report represents a portion of a thesis submitted in partial fulfilment by Kamal Asgar for the degree of Doctor of Philosophy, H. H. Rackham School of Graduate Studies, University of Michigan.

Received for publication April 11, 1960; revised by authors August 18, 1960.

split brass mold, and, by means of a hydraulic wax injector, the molten wax was squirted into the brass mold. After a short time, the wax in the brass mold was solidified, the two blocks were separated, and the wax patterns were removed. The wax pattern and manner of spruing are shown in Figure 2.

In this study a phosphate-bonded investment was used.* The wax patterns were

* Ransom and Randolph's investment No. 711 was used.

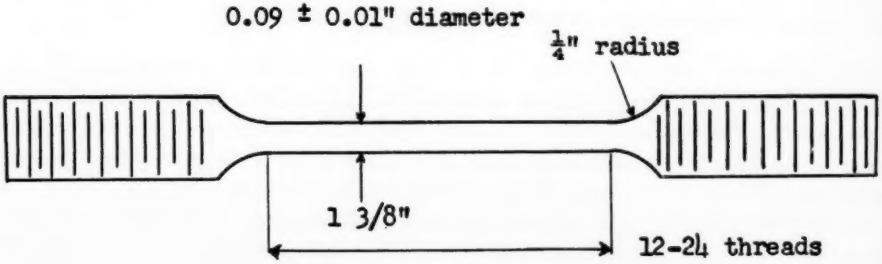


FIG. 1.—Dimensions of test bars

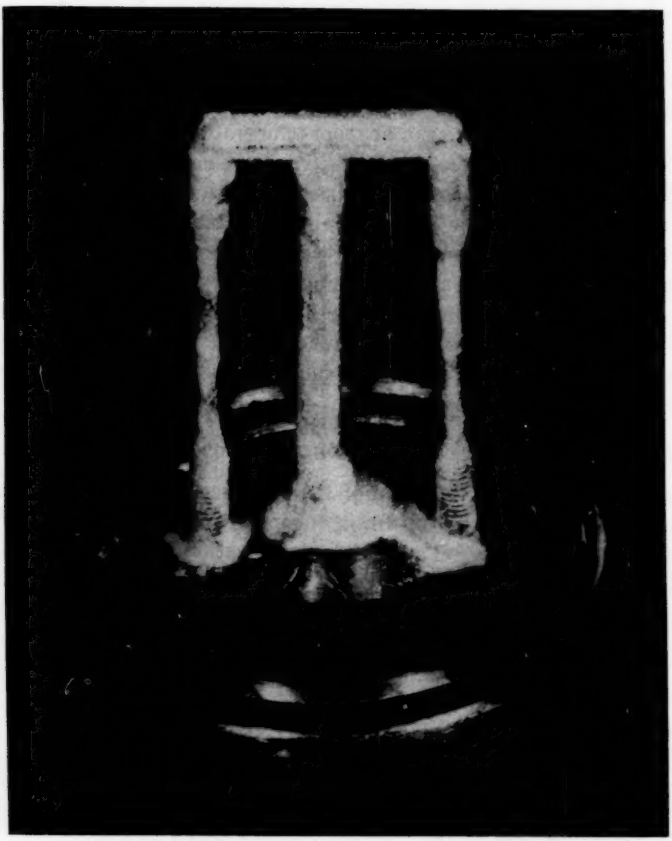


FIG. 2.—Manner of spruing wax pattern

coated with an organic silicate, and a phosphate-bonded investment was used for backing up the precoat. In this study the dimensions of the casting were not important; therefore, the expansion of the investment received little attention. This investment was selected because it produces a clean surface and is relatively inexpensive.

The metal was melted electrolytically, and the temperature of the molten metal in each melt was checked by means of an optical pyrometer.* By having the optical pyrometer mounted on the casting machine, it was necessary to focus the pyrometer only once to direct it properly to cover the field of the crucible. This casting machine is shown in Figure 3, with an optical pyrometer mounted in position. After the casting was completed, the mold was left to cool to room temperature in air. The mold was then broken open and the casting recovered. By means of a sand-blasting unit, the investment particles were removed, and the cast metal piece cleaned. The test bars then were cut off and tested for mechanical properties.

Methods of mechanical testing.—The diameter of each test bar was measured with

*William's Inductocast unit was used for melting and casting of the metal, whereas Leeds and Northrup optical pyrometer was used to check the temperature of each melt.

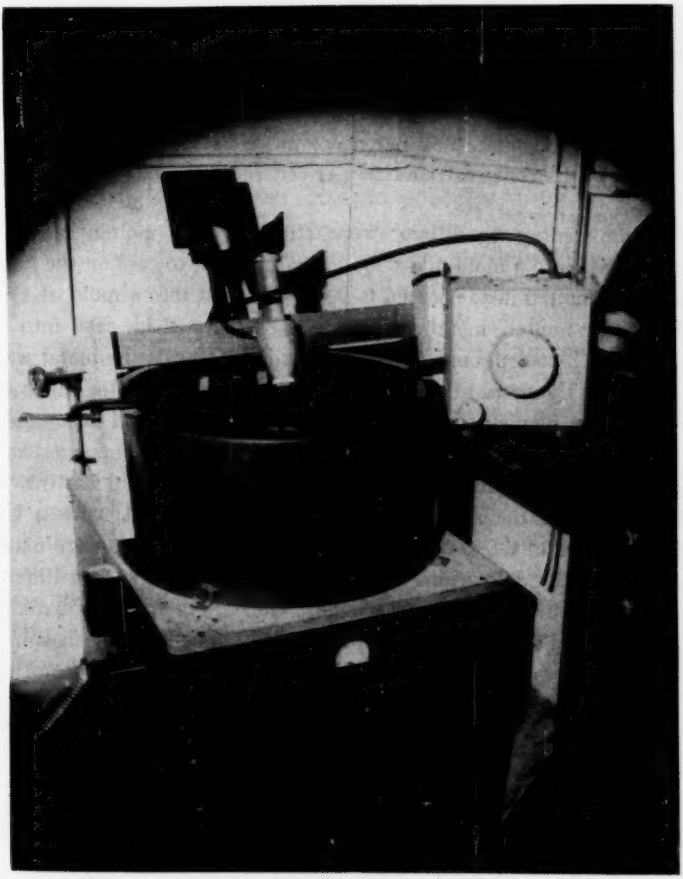


FIG. 3.—Williams casting machine and optical pyrometer

a micrometer, and the values recorded. In order to obtain values for elongation, a distance close to 1 inch was marked on the specimens. By means of a traveling microscope, the distance between these two marks was measured to a hundredth of a millimeter. After the fracture, the test bars were placed as close together as possible, the dimensions between the two marks measured, and the percentage of elongation of the test bars calculated.

The specimens were tested by means of a tensile testing machine employing a head speed of 0.02 inch per minute. To obtain the strain on the sample introduced by the stress, either optical strain gauges or 3M-extensometer was used.* After fracturing and measuring the distance between the two marks for the elongation values, the fractured pieces were mounted in a plastic type of material for metallographic examination. Half of each test bar was mounted and polished through different grades of papers and polishing wheels. The specimens were then etched to reveal their microstructure. In this way the microstructure of specimens with known physical properties was studied. The results of the microstructure studies are being reported separately.[4]

Casting procedure.—Stellite 21 was chosen as a cobalt-base alloy to be used throughout this study. The metal is typical of cobalt-base alloys and resembles closely those high-melting alloys used in dentistry. Since this study is basic in nature, probably similar results, with some minor modifications, can be expected from any one of the alloys of the group designed for dental application. The chemical analysis of Stellite 21 alloy used in this study was carried on, and the results were as follows: chromium, 27.44 per cent; molybdenum, 5.50 per cent; nickel, 2.50 per cent; manganese, 0.64 per cent; iron, 1.74 per cent; carbon 0.25 per cent; silicon, 0.62 per cent; and the balance cobalt.

The following casting conditions were studied: (1) molten metal heated to 2,600° F., then cast into a mold of 1,600° F. and allowed to cool on the bench to room temperature; (2) molten metal heated to 2,600° F., cast into a mold of 1,300° F. and bench-cooled; (3) molten metal heated to 2,800° F., then cast into a mold of 1,600° F., and bench-cooled; and (4) under condition like 1, the metal was heated to 2,600° F., cast into the mold at 1,600° F., and bench cooled, after which the castings were heat-treated by placing them in an oven of 1,600° F. for 1 hour and quenching in water.

Under each one of the above-mentioned four conditions, 20 casting were made. These castings were of the regular size indicated in Figure 1, with a length of $1\frac{3}{8}$ inches and a diameter of 0.090 ± 0.01 inch. Some more castings were made with different specimen lengths. In varying the length of the test bars, four different lengths were arbitrarily chosen: $1\frac{1}{4}$, $\frac{3}{8}$, $\frac{1}{4}$, and $\frac{1}{8}$ inches. Clusters of four test bars, one of each length, were made and attached to one sprue. In this way, all four sizes in the cluster were cast at the same time from one melt. In all these clusters, the metal temperature was 2,600° F., and the mold temperature 1,600° F. A total of five clusters was cast in this manner. These series are represented in Table 1 as conditions 5, 6, 7, and 8. Since the results obtained from condition 3—i.e., metal at the temperature of 2,800° F. cast into the mold at 1,600° F.—showed some difference in values, 20 more specimens with still higher metal temperature were cast. In this series, the metal temperature

* Tuckerman optical gauges and Riehle 3M-extensometer were used in this study.

was 3,000° F., and the mold temperature was 1,600° F. This series is represented in Table 1 as condition 9.

The nine different conditions studied are listed in Table 1; results are summarized and reported in Table 2. This table shows the ultimate tensile strength, yield strength, elongation, and modulus of elasticity values, as well as standard deviations and other values obtained from statistical evaluation of the data.

RESULTS AND DISCUSSION

Ultimate tensile strength.—As can be seen in Table 2, the average ultimate tensile strength of samples cast at 2,600° F. into a mold at 1,600° F. and bench-cooled was 99,600 lb/sq in. When the metal was heated to 2,800° F. and cast into a mold heated to 1,600° F., the average ultimate tensile strength was 95,000 lb/sq in. Subsequent heat treatment of the samples cast at 2,600° F. into a 1,600° F. mold gave ultimate tensile values of 107,400 lb/sq in. Samples cast at 3,000° F. into a mold at 1,600° F.

TABLE 1

CASTING CONDITIONS

Condition	Length of Specimen (Inches)	Metal Temperature (° F.)	Mold Temperature (° F.)	Manner of Cooling
1	1⅜	2,600	1,600	Bench-cooled
2	1⅜	2,600	1,300	Bench-cooled
3	1⅜	2,800	1,600	Bench-cooled
4	1⅜	2,600	1,600	Heat-treated 1 hr. at 1,600° and quenched
5	1¼	2,600	1,600	Bench-cooled
6	¾	2,600	1,600	Bench-cooled
7	¼	2,600	1,600	Bench-cooled
8	⅛	2,600	1,600	Bench-cooled
9	1⅜	3,000	1,600	Bench-cooled

produced an average of 105,500 lb/sq in for the ultimate tensile values. In all these tests, conditions 1, 2, 3, 4, and 9, the tensile test sample size was the same—1⅜ inches—but the casting conditions varied. Although there was some difference in the ultimate tensile strength values obtained, ranging from approximately 96,000 to 107,000 lb/sq in, no single casting condition was superior to another, as indicated by the sizable standard deviation existing under each casting condition. Analysis of variance, which was used to distinguish any difference which might have existed between two sets of results, showed that the difference in values obtained for different test conditions was not significant.

The results of ultimate tensile strength tests on samples of different lengths cast as clusters (conditions 5, 6, 7, and 8) show that values for specimens within each cluster were more uniform than the values obtained from different clusters of test samples. The results from individual test samples of one cluster of four, for example, gave ultimate tensile strength values of 114,400, 112,300, 113,900, and 112,200 lb/sq in for specimens with a length of 1¼, ¾, ¼, and ⅛ inches, respectively. A second cluster of test samples gave ultimate tensile strength values of 102,000, 104,000, 90,900, and 89,600 lb/sq in, respectively, for the four lengths that were cast. All four specimens in this

TABLE 2

RESULTS OF THE NINE CASTING CONDITIONS

	Ult. Tensile (#/in²)	0.1 Per Cent Yield (#/in²)	0.2 Per Cent Yield (#/in²)	Mod. Elast. (×10⁶)	Elongation (Per Cent)
	Condition 1				
Average	99,650	75,760	80,870	33.7	4.12
S.D.	6,900	2,360	2,950	1.55	
95% confidence interval	114,380	80,800	87,200	37.00	Max. 7.4
	84,920	70,800	74,600	30.40	Min. 1.7
	Condition 2				
Average	100,100	75,600	81,030	34.13	3.38
S.D.	3,850	2,400	2,918	2.48	
95% confidence interval	108,300	80,700	87,200	39.24	Max. 8.00
	91,900	70,500	74,800	29.01	Min. 1.48
	Condition 3				
Average	95,900	72,820	76,030	33.45	6.94
S.D.	4,600	2,166	2,608	1.65	
95% confidence interval	106,300	77,700	81,900	37.13	Max. 12.90
	85,500	67,900	70,100	29.77	Min. 3.42
	Condition 4				
Average	107,400	69,960	78,230	36.85	3.37
S.D.	7,750	1,530	1,920	1.03	
95% confidence interval	124,900	73,400	82,600	39.2	Max. 4.47
	89,900	66,500	73,900	34.5	Min. 1.73
	Condition 5				
Average	103,100	79,000	81,900	38.5	3.1
S.D.	8,950	3,575	2,980	2.5	
95% confidence interval	126,200	88,500	89,500	44.9	Max. 6.4
	80,000	70,000	74,300	32.1	Min. 0.5
	Condition 6				
Average	107,600	.....	.....	.....	4.75
S.D.	6,300	.....	.....	.....	
95% confidence interval	125,100	.....	.....	.....	Max. 7.5
	90,100	.....	.....	.....	Min. 1.0
	Condition 7				
Average	104,600	.....	.....	.....	9.6
S.D.	7,750	.....	.....	.....	
95% confidence interval	129,700	.....	.....	.....	Max. 14.0
	79,500	.....	.....	.....	Min. 6.2

TABLE 2—*Continued*

	Ult. Tensile (#/in²)	0.1 Per Cent Yield (#/in²)	0.2 Per Cent Yield (#/in²)	Mod. Elast. (×10⁶)	Elongation (Per Cent)
	Condition 8				
Average	99,300	.....	.....	.....	11.3
S.D.	8,000	.....	.....	.....	.....
95% confidence interval	119,900	.....	.....	.....	Max. 15.6
	78,700	.....	.....	.....	Min. 8.2
	Condition 9				
Average	105,500	77,100	81,500	36.1	7.6
S.D.	4,950	2,650	3,240	2.1	.....
95% confidence interval	118,200	83,900	89,800	41.4	Max. 9.9
	92,800	70,300	73,200	30.8	Min. 5.6

cluster had somewhat lower values than those of the first cluster, regardless of the sample length. Since results of only five clusters are available, however, it is not safe to draw definite conclusions, except that it appears probable that the unknown factors which cause variations in values of a cluster of samples are more effective than the specimen length on the ultimate tensile strength value.

The values obtained in this study were in close agreement with the values obtained by other investigators. Earnshaw[5] reported, in 1956, the tensile strength values of some English-made cobalt-base alloys used in dentistry, in which the ultimate tensile strength of seven dental alloys tested had a range of 96,000–120,000 lb/sq in. His samples were 0.178 inch in diameter and 0.631 inch in length, which is different from the size used in this study. Earnshaw's values were reported as average values for each alloy type, and the individual variations in his results in each group were not discussed. More recently Taylor, Leibfritz, and Alder[6] reported the ultimate tensile strength values of different dental alloys, with results varying from 85,000 to 108,000 lb/sq in, and the standard deviation of different groups ranging from 4,000 to 12,000 lb/sq in. Taylor *et al.* used the same specimen size as was used in this study. Their results and the results obtained in this study for the ultimate tensile strength and its variations agree quite satisfactorily.

The values published by Haynes Stellite Company[7] are given as an average value of 101,300 lb/sq in at room temperature for the ultimate tensile strength of Stellite 21. Although the test bars used by the Haynes Stellite Company had a diameter of 0.250 inch, whereas in this study they were only 0.090 ± 0.01 inch, the values obtained for the ultimate tensile strength show close agreement.

It seems safe to assume, therefore, that the ultimate tensile strength of Stellite 21 is approximately 100,000 lb/sq in and that the size of the test specimen, as well as the casting conditions, may not have a significant effect on this property. Examination of the microstructure of different specimens revealed that even specimens containing a small amount of microporosity had an ultimate tensile strength as high as the specimens free of microporosity. It should be emphasized that this may possibly be true

only for the ultimate tensile strength and not necessarily for other properties. As an example, photomicrographs of specimens Nos. 80 and 87 are shown in Figures 4 and 5. The ultimate tensile strengths of these specimens were both equal to 97,400 lb/sq in, even though the photomicrograph of specimen 87 shows some microporosity and that of 80 is free of porosity. The fact that specimens with some microporosity still show a comparatively high ultimate tensile strength may be due to the high modulus of elasticity and the high yield point of the alloy.

Yield strength and elastic modulus.—In order to obtain values of 0.1 per cent yield strength, 0.2 per cent yield strength, and modulus of elasticity, the relationship between stress and strain should be known. In general, no problem exists in obtaining the stress value on the sample at any instant during the testing. It is more difficult, however, to obtain the values for strain. Most values were obtained in this study by the use of the 3M-extensometer, which was a non-averaging, magnetic snap-on type.

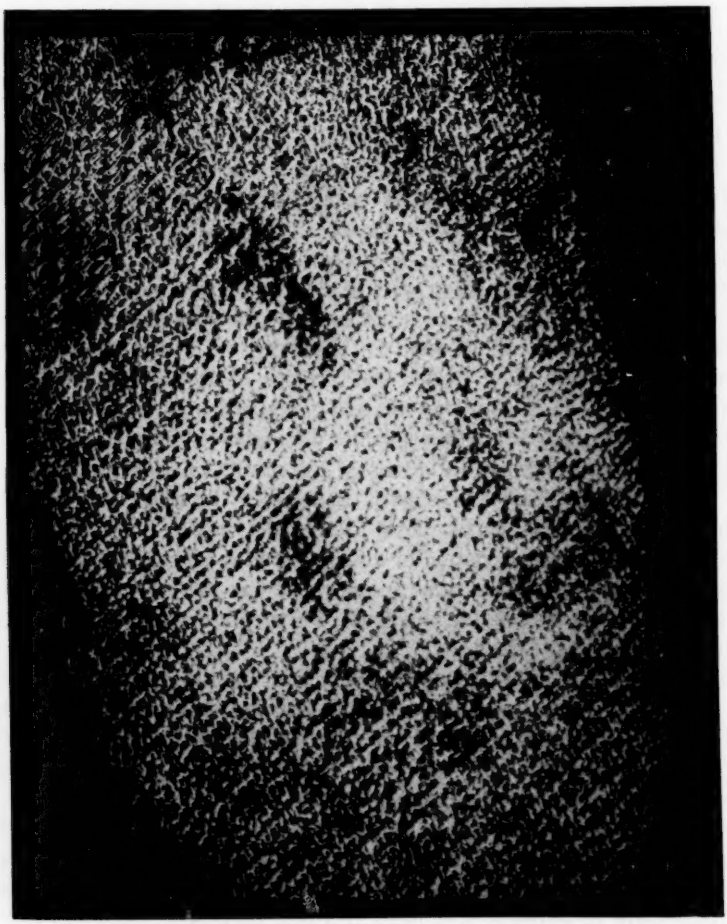


FIG. 4.—Microstructure of specimen 80. (Mag. ×85.)

This type of extensometer is quite convenient to use, but, since it is a non-averaging type and the samples were small, extreme care is necessary in preparing the samples.

The sensitivity of the 3M-extensometer and optical strain gauges and the validity of the results obtained were checked against a known sample. An aluminum specimen with a $\frac{1}{4}$-inch diameter and a modulus of elasticity known to be 10.6×10^6 lb/sq in was machined, and the results were obtained from optical strain gauges, as well as the 3M-extensometer. The values obtained from optical strain gauges were 10.1×10^6 and 10.9×10^6 lb/sq in, with the average of 10.5×10^6 lb/sq in, and that obtained by the 3M-extensometer was 10.6×10^6 lb/ sq in. These results agree with the modulus of the elasticity of the aluminum sample.

The relation of strain to stress will vary if the specimen is bent during preparation or testing. This variation will increase if the diameter of the specimen is small or if the material tested has a low magnitude of strain. Both these factors were found to be

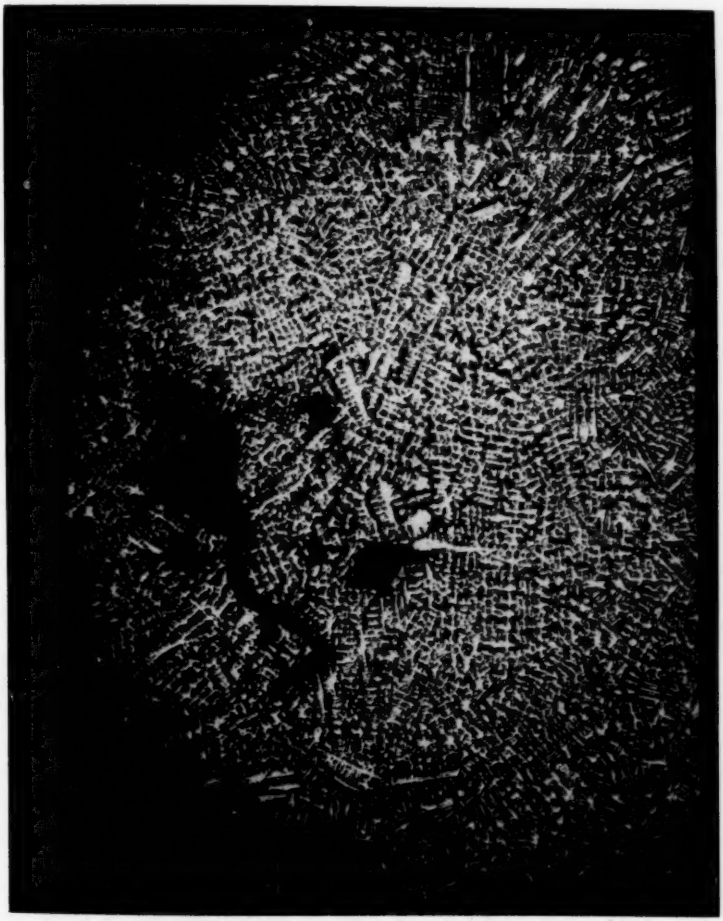


FIG. 5.—Microstructure of specimen 87. (Mag. ×85.)

effective in obtaining the stress-strain relationship in this study. Templin[8] has shown that surface conditions along the gauge length of the specimen, as well as the eccentric loading applied to the specimen, which results in a bending moment, may vary the stress-strain relationship considerably. Although extreme care was exercised in preparing the samples and performing the test, variation in the modulus of elasticity continued to be observed, a condition which is contrary to the principle that the modulus of elasticity of a material should be constant and not affected by heat treatment, variation of casting conditions, or other factors of manipulation. Theoretically, the only way to change the modulus of elasticity of an alloy is by altering its chemical composition. Consequently, the variation in the modulus of elasticity in this study should not be attributed to poor testing technique or the size of the sample. Similar results have been obtained by other investigators. Taylor *et al.*[6] have reported their modulus of elasticity value to range from 28×10^6 to 31.0×10^6 lb/sq in for each alloy tested, and the standard deviation was reported as 2.5×10^6 lb/sq in. The results obtained for the modulus of elasticity by other investigators[9] using specimens $\frac{1}{4}$ inch or $\frac{1}{2}$ inch in diameter have also shown similar variations. In this study the calculated standard deviation for different conditions varied from 1.03×10^6 to 2.5×10^6, with average values ranging from 33.45 to 38.5×10^6 lb/sq in.

It is possible to obtain 0.1 and 0.2 per cent yield strength values from the stress-strain curve. It is somewhat more convenient and practical to obtain the offset yield strength than the value for proportion limit. The values of 0.1 and 0.2 per cent yield strength obtained under condition 4 were somewhat lower than those obtained under other conditions. The 0.1 per cent yield strength of 75,000 lb/sq in or higher was obtained in other conditions, whereas the 0.1 per cent yield strength value for condition 4 averaged 69,960 lb/sq in. The yield strength of the alloy is important in practice, since it represents the value where permanent deformation takes place in the structure. If an alloy has a high modulus of elasticity but a low yield strength, it is not a proper alloy for partial dentures. Alloys used in partial dentures should have not only a high modulus of elasticity but also a high yield strength, so that permanent deformation of the cast piece does not take place easily. Griffiths[10] points out that the minimum yield strength of cobalt-base alloys used in dentistry should be 70,000 lb/sq in.

Because of the short specimen size (less than 1 inch), the stress-strain relationship was not obtained for conditions 6, 7, and 8, and, as a result, the 0.1 and 0.2 per cent yield strength and the modulus of elasticity are not reported for these test conditions.

Elongation.—One of the important properties of a dental alloy is its ductility, which in most cases is indicated as a percentage of elongation. In burnishing the gold inlays or in adjusting the clasps on partial dentures, the ductility of the cast pieces is most important. Both before initial insertion and after being in service for some time, partial denture clasps may need some adjustments, for which reason the ductility of the cast piece becomes an important property. The test specimen is marked along its length, and the distance between the two gauged marks is measured before and after the fracture. The change in the length of the specimen is reported on a percentage basis, as per cent of elongation, which gives some indication of the ductility of the material. This value was obtained for all specimens

but for conditions 6, 7, and 8 and is reported in Table 2. In contrast to the ultimate tensile strength values, this value varied from a very low value of 0.5 to as much as 12 per cent. The presence of microporosity had a definite effect on the per cent of elongation.

It was found that, in order for the castings of this alloy to have higher elongation values, not only should they be sound and free from microporosity but also their microstructure should be of a definite type. To clarify this statement, consider the physical properties as well as the microstructures of specimens 80 and 87, which are shown in Figures 4 and 5. Both specimens were cast under the same conditions, and both registered an ultimate tensile strength of 97,400 lb/sq in, whereas the per cent of elongation of specimen 80 was 8.25 per cent, and that of specimen 87 was only 3.4 per cent. The microstructure of specimen 87 reveals the presence of some microporosities, whereas the microstructure of the specimen 80 was free of any porosities. These findings indicate that the presence of microporosities is an important factor in elongation values. Comparing the photomicrographs of specimens 51 and 58 shown in Figures 6 and 7, it is seen that, although both these specimens were cast under the same conditions, they possess different microstructures. Specimen 51 shows a very slight amount of microporosity and a 6.6 per cent elongation, whereas specimen 58, with no microporosity, had only 2.1 per cent elongation. This indicates that microstructure may be as significant in modifying the properties as is the microporosity. This part of the study is discussed in more detail in a separate publication.[4]

Examination of the results obtained from condition 3 reveals that, in general, the specimens of this group have relatively high elongation values. Specimens without microporosities showed as much as 12 per cent elongation, and the specimens with microporosities showed over 3 per cent elongation. In Table 2 the average values for the per cent of elongation, using the full length of the test samples as a gauge length, were 4.12, 3.38, 6.94, 3.37, and 7.6. The two higher values, 6.94 and 7.6 per cent, represent the metal casting temperatures of 2,800° F. and 3,000° F. cast into molds of 1,600° F. When this metal was heated to 3,000° F. and cast, the interaction between the surface of the metal and the mold material was great. Because of the reaction of metal with mold material, the surface condition of the cast pieces was poor and not acceptable in practical dental cases. At low metal temperatures such as 2,600° F., the surfaces of the castings were clean, and there were no interphase reactions between the metal and the investment, but the average elongation was 3–4 per cent. At a metal temperature of 2,800° F., the metal surface was not damaged; however, it was somewhat darker gray in color, and the average elongation was about 7 per cent. The reduction in a cross-sectional dimension (diameter) of a test sample cast at 2,800° F., with an elongation of 7.25 per cent, is shown in Figure 8. It can be seen from Figure 8 that the cobalt-base alloys, when cast under certain conditions, may possess higher elongation values than are usually known in dental castings.

The averages of the elongation values reported for conditions 5, 6, 7, and 8 were 3.1, 4.75, 9.6, and 11.3 per cent, respectively. These values were expected to be the same, since the mold and metal temperature for conditions 1, 5, 6, 7, and 8 were identical. It should be recalled that the lengths of the test samples for specimens

whose results were reported in Table 2 under conditions 6, 7, and 8 were $\frac{3}{4}$, $\frac{1}{4}$, and $\frac{1}{8}$ inch but all had a diameter of 0.097 inch. The elongation took place mainly in the thin section of the specimen between the threads, and the per cent of elongation was calculated on this basis. It is well known that if the elongation of a sample is based on shorter gauge marks and if the fracture takes place between the marked gauges, the calculated per cent of elongation is much greater than if a longer gauge length is used. The higher elongation reported for these specimens was probably due to a shorter gauge length.

SUMMARY AND CONCLUSIONS

A total of 120 test specimens of Stellite 21 were cast under different casting conditions. The conditions were mainly changing metal temperature, altering the mold temperature, heat-treating the test specimens, as well as varying the length of the specimen. Tensile strength, 0.1 and 0.2 per cent yield strength, modulus of elasticity,

FIG. 6.—Microstructure of specimen 51. (Mag. ×800.)

FIG. 7.—Microstructure of specimen 59. (Mag. ×800.)

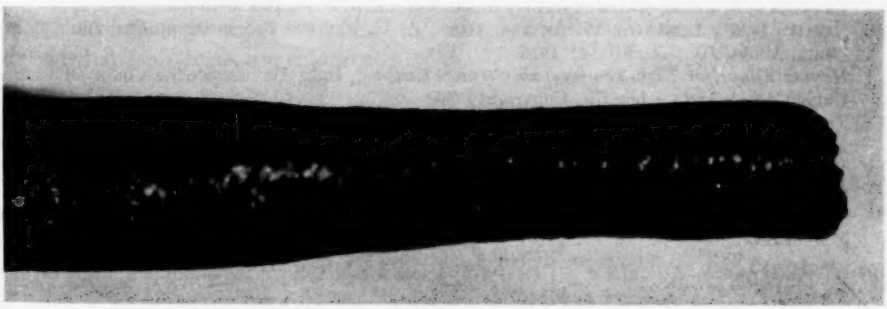


FIG. 8.—Reduction in cross-sectional area of the specimens with higher elongation values

and per cent of elongation for the test bars were obtained. The results were statistically analyzed, and the average, standard of deviation, and 95 per cent confidence interval for each group were calculated. From the results of this study, the following statements may be drawn.

The ultimate tensile strength of Stellite 21 at room temperature was found to be approximately 100,000 lb/sq in, which varied little with a change in metal casting temperature, mold temperature, and the length of the test specimen.

The presence or absence of microporosities in the alloy did not change the ultimate tensile strength significantly. The presence of microporosities, as well as different types of microstructure in the specimens, has a significant effect on the elongation values obtained.

The values obtained for 0.1 per cent and 0.2 per cent yield strengths were more uniform than the ultimate tensile strength values.

The modulus of elasticity of Stellite 21 under testing conditions of this study varied between approximately 30 and 40 $\times$ 10^6 lb/sq in, with the typical value being 35 $\times$ 10^6 lb/sq in.

Specimens with metal casting temperatures of 2,800° F. and 3,000° F. yielded a higher per cent elongation than when a lower temperature was used. At 3,000° F. metal casting temperature, however, the interphase reaction between the surface of the metal and the investment increased considerably, and, as a result, the surfaces of the cast pieces were damaged.

The chosen heat-treating cycle did not improve any of the properties of the alloy and possibly harmed them. The surface appearance of the heat-treated pieces was somewhat inferior.

REFERENCES

1. Lane, J. R. A Survey of Dental Alloys, *J.A.D.A.*, **39**:441, 1949.
2. Bush, S. H., Ingersoll, C. H., and Peyton, F. A. *The Effect of Section Size on the Mechanical Properties of Cast Chrome-Cobalt Dental Alloys.* Navy Contract NG-ONR-232 Progress Report, School of Dentistry, University of Michigan, 1952.
3. Taylor, D. F., and Sweeney, W. T. A Proposed Specification for Dental Chromium-Cobalt Casting Alloys, *J.A.D.A.*, **54**:44, 1957.
4. Asgar, K., and Peyton, F. A. The Effect of the Microstructure on the Physical Properties of Chromium-Cobalt Alloys, *J. D. Res.*, in press.
5. Earnshaw, R. Cobalt-Chromium Alloy in Dentistry, *Brit. D. J.*, **101**:67, 1956.
6. Taylor, D. F., Leibfrita, W. A., and Alder, A. G. Physical Properties of Chromium-Cobalt Dental Alloys, *J.A.D.A.*, **56**:343, 1958.
7. *Haynes Alloys for High-Temperature Service.* Kokomo, Ind.: Haynes Stellite Co., a Division of Union Carbide and Carbon Co., Copyright 1950.
8. Templing, R. L. Effect of Size and Shape of Test Specimens on the Tensile Properties of Sheet Metals, *A.S.T.M. Proc.*, **26**:378, Part II, 1926.
9. "Heat-resistant Casting, by Subcommittee on Heat-resistant Castings." In T. Lyman (ed.), *Metal Handbook*, p. 570. Cleveland: Amer. Soc. Met., 1948.
10. Griffiths, E. S., and Cashman, W. D. Alloy Adapted for Prosthetic Articles. U.S. Patent 2,631,095. March, 1953.

Effect of Dietary Casein-Sucrose Ratios on Amino Acid Composition of Dentinal Protein

W. C. HESS, A. DHARIWAL, J. F. CHAMBLISS, JR., and Z. C. ALBA
Georgetown University Schools of Medicine and Dentistry, Washington, D.C.

Losee, Van Reen, Peckham, Hess, Henderson, and Gerende[1] maintained rats on diets containing 20, 30, and 40 per cent casein, designated 20P, 30P, and 40P. All the rats on all the diets developed carious lesions. While there were no marked differences in the caries responses to any of the 3 diets, there were differences in weight gain. Maximum body weight gain was on the 30 per cent casein diet. The proteins from the incisor dentin were isolated and their amino acid composition was determined by microbiological assay. The error inherent in these methods was felt to be of an order of magnitude that deprived the variations in composition of any significance.

The analyses of these proteins have been repeated by the precise chromatographic methods devised by Moore and Stein.[2] Hughston, Earle, and Binkley[3] have used one of the Moore and Stein methods for the analysis of the protein isolated from human dentin. We have included an analysis of human dentinal protein by the chromatographic method for the purpose of comparison with their results and also with the earlier results of Hess, Lee, and Neidig.[4]

MATERIALS AND METHODS

The human dentinal protein was the same sample as that previously analyzed.[4] It had been prepared by dialytic demineralization against 0.1 N HCl. Proteins had been isolated from the incisor dentin of the rats on the 3 different casein diets by demineralization with EDTA (ethylenediaminetetraacetate) at pH 7.5.[1] The proteins were hydrolyzed by 6 N HCl for 24 hours in a sealed tube.

The amino acid analyses were done by the most recent method of Moore, Spackman, and Stein,[5] using a polystyrene resin.* The hydraulic separation method of Hamilton[6] was used for the preparation of the resin particles of appropriate size.† The colorimetric ninhydrin procedure of Moore and Stein[7] was used and the optical densities were determined in a photoelectric colorimeter.

RESULTS AND DISCUSSION

The results of the analyses of human dentinal protein are given in Table 1, together with the earlier results of Hess *et al.*[4] and also those of Binkley.[3] The total nitrogen content of each protein was determined by the micro-Kjeldahl method. The values for

This study was supported by Research Grant D-570 from the N.I.H., U.S. Public Health Service.
Received for publication April 25, 1960; revised by authors June 13, 1960.

* Amberlite IR-120, Fisher Scientific Co.

† This step was very kindly conducted for us by Dr. K. A. Piez at the National Institutes of Dental Research.

each protein are included, together with those for the amino acids. The recovery of nitrogen in the amino acids and ammonia estimated is given in the adjacent column. The final figures are the per cent recovery of the total nitrogen. No marked differences were noted in the values for any of the amino acids except hydroxylysine. Our hydroxylysine values are about half those of Binkley but are of the same order as previously found by Battistone and Burnett.[8] We found no evidence for the existence of the peptides in the hydrolysate of the dentin that were reported by Binkley.[3] The temperature for hydrolysis used was between 105° and 110° C. rather than their temperature of

TABLE 1*

AMINO ACID COMPOSITION OF HUMAN DENTINAL PROTEIN

	New (Per Cent)		Hess† (Per Cent)		Binkley‡ (Per Cent)
	Amino Acid (1)	N (2)	Amino Acid (3)	N (4)	Amino Acid (5)
Alanine	8.0	1.26	8.5	1.34	6.0
Aspartic	5.7	0.60	5.6	0.59	5.6
Arginine	7.8	2.50	7.4	2.38	7.5
Cystine	0.1	0.01	0.1	0.01	0.0
Glutamic	10.0	0.95	10.6	1.01	9.3
Glycine	22.4	4.18	22.5	4.20	20.2
Histidine	0.8	0.22	0.8	0.22	0.7
Hydroxylysine	1.3	0.14	1.0	0.11	2.7
Hydroxyproline	14.8	1.58	13.1	1.40	15.7
Isoleucine	1.4	0.15	1.3	0.13	1.1
Leucine	3.8	0.41	3.6	0.38	2.7
Lysine	3.4	0.65	3.3	0.64	2.6
Methionine	0.5	0.05	0.5	0.05	0.7
Phenylalanine	2.0	0.18	1.9	0.16	2.1
Proline	13.5	1.64	16.3	1.98	13.0
Serine	3.5	0.45	3.5	0.45	3.6
Threonine	2.1	0.25	3.1	0.36	1.5
Tyrosine	1.0	0.08	1.1	0.09	0.5
Tryptophan	0.0	0.0	0.0	0.0	0.0
Valine	2.2	0.26	3.0	0.36	2.3
Ammonia	0.5	0.41	.....	.....	0.6
Nitrogen	16.15	15.97	16.15	15.86	17.9
Per cent recovery of N	.....	98.8	.....	98.2	98.4

* The values in cols. 1 and 3 are expressed as grams of amino acid per 100 gm. protein. The values in cols. 2 and 4 are the amount of nitrogen in each amino acid per 100 gm. protein. The values in col. 5 are residue weights and need to be increased by 10–15 per cent to be comparable to those in cols. 1 and 3.

† See ref. 4. ‡ See ref. 3.

100° C. We have never failed to obtain complete hydrolysis of the many proteins previously analyzed under our own conditions.

The data on the three proteins from the rats fed 20P, 30P, and 40P are given in Table 2. No significant differences were found in the composition of the three proteins. The values were substantially the same as those found for human dentin. It is apparent that the variations in the protein content of the diet had no effect on the composition of the dentin protein.

SUMMARY

Polystyrene resin was used for the chromatographic analysis of the proteins isolated from human dentin and from the incisors of rats fed diets containing 20, 30, and 40

per cent casein. The amino acid composition of the human dentin protein determined by this precise method was similar to that previously found by other methods of analysis and by other investigators. The rat incisor dentinal proteins from the three diets showed no differences one from the other and closely resembled the human dentinal protein in amino acid composition.

TABLE 2*

AMINO ACID COMPOSITION OF RAT DENTINAL PROTEINS

	20P		30P		40P	
	Amino Acid (Per Cent) (1)	N (Per Cent) (2)	Amino Acid (Per Cent) (3)	N (Per Cent) (4)	Amino Acid (Per Cent) (5)	N (Per Cent) (6)
Alanine	5.9	0.92	6.4	1.01	5.9	0.92
Aspartic	5.8	0.61	5.4	0.57	5.4	0.57
Arginine	6.6	2.12	6.8	2.19	7.0	2.25
Cystine	0.1	0.01	0.1	0.01	0.1	0.01
Glutamic	8.6	0.82	8.2	0.78	8.2	0.78
Glycine	20.6	3.84	20.8	3.88	21.0	3.92
Histidine	0.9	0.24	1.0	0.27	0.7	0.19
Hydroxylysine	1.4	0.15	0.9	0.10	0.9	0.10
Hydroxyproline	14.3	1.53	14.0	1.50	13.2	1.41
Isoleucine	1.1	0.12	1.1	0.12	1.2	0.13
Leucine	4.0	0.43	3.7	0.40	3.6	0.38
Lysine	4.0	0.77	3.8	0.73	3.6	0.69
Methionine	0.5	0.05	0.5	0.05	0.5	0.05
Phenylalanine	2.3	0.20	1.8	0.15	2.0	0.17
Proline	13.3	1.62	12.8	1.56	12.8	1.56
Serine	3.0	0.40	3.2	0.43	3.7	0.49
Threonine	1.8	0.21	1.8	0.21	2.2	0.26
Tyrosine	1.0	0.08	1.0	0.08	1.0	0.08
Tryptophan	0.0	0.0	0.0	0.0	0.0	0.0
Valine	2.8	0.33	3.3	0.39	3.3	0.39
Ammonia	0.5	0.41	0.5	0.41	0.5	0.41
Nitrogen	15.1	14.86	15.6	14.84	15.4	14.76
Per cent of recovery of N	.....	98.4	.....	95.1	.....	95.8

* The values in cols. 1, 3, and 5 are expressed as grams of amino acid per 100 gm. protein. The values in cols. 2, 4, and 6 are the amounts of nitrogen in each amino acid per 100 gm. protein.

REFERENCES

1. LOSEE, F. L., VAN REEN, R., PECKHAM, S. C., HESS, W. C., HENDERSON, N., and GERENDE, L. J. Dietary Casein-Sucrose Ratios and Their Effects on Mineralized Tissue, *J. D. Res.*, **36**:904, 1957.
2. MOORE, S., and STEIN, W. H. Chromatography of Amino Acids on Sulfonated Polystyrene Resins, *J. Biol. Chem.*, **192**:663, 1951.
3. HUGHSTON, H. H., EARLE, L. S., and BINKLEY, F. Amino Acid Composition of Proteins of Human Dentin, *J. D. Res.*, **38**:323, 1959.
4. HESS, W. C., LEE, C., and NEIDIG, B. A. Amino Acid Composition of Dentin Protein, *Proc. Soc. Exptl. Biol. Med.*, **76**:783, 1951.
5. MOORE, S., SPACKMAN, D. H., and STEIN, W. H. Chromatography of Amino Acids on Sulfonated Polystyrene Resins, *Anal. Chem.*, **30**:297, 1958.
6. HAMILTON, P. B. Ion-Exchange Chromatography of Amino Acids: Effect of Resin Particle Size on Column Performance, *Anal. Chem.*, **30**:914, 1958.
7. MOORE, S., and STEIN, W. H. Modified Ninhydrin Reagent for the Photometric Determination of Amino Acids and Related Compounds, *J. Biol. Chem.*, **211**:907, 1954.
8. BATTISTONE, G. C., and BURNETT, G. W. Studies of the Composition of Teeth. III. The Amino Acid Composition of Human Dentinal Protein, *J. D. Res.*, **35**:255, 1956.

A Stannous Fluoride, Silex, Silicone Dental Prophylaxis Paste with Anticariogenic Potentialities

VINCENT A. SEGRETO, NORMAN O. HARRIS, and WARREN R. HESTER

Department of Dental Science, School of Aviation Medicine, USAF, Brooks Air Force Base, Texas

The incorporation of an effective anticariogenic agent into a dental prophylaxis paste would insure the patient's receiving a simultaneous caries-preventive treatment with each cleaning and polishing operation. In addition, the simplicity of the treatment would increase the utility of the topically applied fluoride prophylaxis. In 1946, Bibby, Zander, McKelleget, and Labunsky[1] reported results of a clinical study directed toward this objective, using a 1 per cent sodium fluoride, pumice, and hydrogen peroxide mixture. A half-mouth control technique was used, and a 43 per cent decrease in caries was noted in 47 children participating. A larger study, which was undertaken to include over 250 patients with three cleanings per year, resulted in no reduction.[2]

More recently, favorable anticariogenic results have been reported following the use of either a single topical application of stannous fluoride or stannous fluoride-containing dentifrices,[3] thereby renewing the possibility of formulating an effective dental prophylaxis paste.

In the following study a stannous fluoride, silex, silicone dental prophylaxis paste was developed to determine: (1) the in vitro effectiveness of the finalized formula of the prophylaxis paste, (2) the effect of the paste when individual components were successively omitted, and (3) the stability of the paste as a function of time.

EXPERIMENTAL METHODS

The intact, non-carious extracted teeth used in this study were secured at various geographic locations, both military and civilian. All teeth were placed in water immediately following extraction, avoiding the need for fixatives or sterilizing agents which might possibly have some action on either the inorganic or the organic components of the enamel.

In all experiments, each replicate (rep) consisted of 4 teeth in each of the experimental and control groups. The teeth selected for each study were either all molars or bicuspids and were chosen in order to present approximately the same surface exposure to subsequent decalcification procedures. Following scaling, the roots of all the teeth were covered with wax from the cervico-enamel junction to the apex.[4] The exposed crowns were then subjected to the experimental or control procedures.

To test the effectiveness of each treatment, all control and experimental teeth were

---

The opinions asserted are those of the authors and are not to be construed as representing the views of the United States Air Force.

Received for publication April 26, 1960, revised by authors September 30, 1960.

individually placed in 2 ml. of 0.1 per cent formic acid for a 24-hour period. One ml. of the supernatant fluid from each tube was then utilized for phosphorus analysis, employing the method of Taussky and Shorr.[5] Data evaluation was based on the assumption that the least phosphorus loss would indicate the most effective treatment.

METHODS

Phase I.—Four replicate experiments were conducted to demonstrate the effectiveness of the stannous fluoride, silex, silicone dental prophylaxis paste. In order to determine the phosphorus loss following no protective treatment, teeth in the first control group were cleaned with silex and water, immersed in triple-distilled water for 5 minutes, and then air-dried. A second control was given a topical application of a 10 per cent solution of SnF_2, an agent known to reduce enamel solubility.[3] Teeth in this group were cleaned with silex and water, air-dried, then immersed in a 10 per cent stannous fluoride solution for 5 minutes and again air-dried. The third group was cleaned with the experimental stannous fluoride, silex, silicone prophylaxis paste.

The experimental prophylaxis paste was formulated as follows: The dry ingredients were silex (ground quartz) SiO_2, mesh, extra-fine;* stannous fluoride, 100 per cent, 40 mesh;† and cyclamate sodium with saccharin, tablet.‡ The liquid ingredients included: Silicone, Dow Corning No. 555 fluid;§ liquid petrolatum, USP (heavy); oil of orange (concentrated); and oil of anise (concentrated).

The following procedure was used for mixing the paste. Two gm. each of silex and stannous fluoride were weighed and mixed. One-fourth of the finely ground sucaryl tablet (approximately 13 mg.) was added, and the ingredients mixed thoroughly. For every 4 gm. of the mixture of dry ingredients the following liquids were added: silicone fluid, 0.1 ml; liquid petrolatum, 0.4 ml.; oil of orange, 0.2 ml.; and one drop of anise oil.

All teeth in the three groups were concurrently placed in individual test tubes containing 2 ml. 0.1 per cent formic acid for 24 hours at 37° C.

Phase II.—A series of successive experiments was conducted to determine the separate effect of the various ingredients of the paste in inhibiting in vitro decalcification. Specifically tested were (*a*) the paste without solid ingredients; (*b*) the paste without silicone (with only liquid petrolatum used as a mostening agent); (*c*) the paste without petrolatum (with only the silicone used as the moistening agent); (*d*) the paste without stannous fluoride; and (*e*) the paste without silex.

Phase III.—In order to determine the optimum stannous fluoride necessary to effect maximal tooth protection to in vitro decalcification procedures, a series of replicate studies was carried out in which the stannous fluoride–silex proportions in the paste ranged from 1:1 to 1:7.

Phase IV.—This series was conducted in order to determine the stability of this nonaqueous dental prophylaxis paste as a function of time. A sufficient quantity was prepared initially to allow weekly replicate testing over a 5-week period. At each period, 4 teeth treated with the paste were compared with the water controls.

* S. S. White Mfg. Co., Philadelphia, Pa.
† Ozark Mahoning Co., 310 W. 6th Street, Tulsa, Okla.
‡ Abbott Laboratories, North Chicago, Ill.
§ Dow Corning, Midland, Mich.

RESULTS

Since the type of tooth (including size) selectively used for each rep was randomly chosen from either stocks of extracted molars or bicuspids, inferences from statistical tests based on the average (averaged over reps) can be made for both these types of teeth, whereas inferences from statistical tests based on a single rep can be made only for the particular type of tooth used in that rep. All testing was done either by analysis of variance or by a multiple comparisons test.

Phase I.—In an analysis of variance, the four replications of the three treatments (water, 10 per cent topical application of SnF_2, and paste) resulted in the paste being significantly more effective in preventing phosphorus loss than either the 10 per cent SnF_2 application ($P>0.05$) or the water ($P>0.01$). The application of the 10 per cent SnF_2 was not statistically different from water (Table 1).

Phase II.—(*a*) In two replicates of two treatments where the paste liquid, i.e., without the silex and the stannous fluoride, was compared to a water control, the effectiveness of the paste liquid against decalcification procedures did not differ from the H_2O (Table 2). (*b*) In the two reps comparing the paste without silicone to the water control, the increase in protection using the paste is not sufficient to draw an inference of effectiveness for all teeth, even though the paste gives a significant increase in protection for the two types of teeth used in the experiment ($P>0.01$) (Table 3). Probably

TABLE 1

EFFECTIVENESS OF SnF_2 PROPHYLAXIS PASTE IN COMPARISON WITH WATER TREATMENT AND TOPICALLY APPLIED 10 PER CENT SnF_2 (MG. PER CENT PHOSPHORUS LOST DURING DECALCIFICATION)

Replicate	H_2O Control	SnF_2 10 Per Cent Topical	SnF_2 Paste
1	0.562	0.362 (35.6*)	0.022 (96.1*)
2	0.648	0.568 (12.3)	0.038 (94.1)
3	0.895	0.698 (21.9)	0.008 (99.1)
4	1.130	0.935 (17.2)	0.082 (92.7)
Average	0.809	0.641 (21.8)	0.038 (95.5)

S.D.$_1$ = 0.268; df = 6. Used to test average treatment differences.
S.D.$_2$ = 0.070; df = 36. Used to test treatment differences for each replicate separately.

TABLE 2

EFFECTIVENESS OF VARIOUS SnF_2 PROPHYLAXIS PASTE COMPONENTS (MG. PER CENT PHOSPHORUS LOST DURING DECALCIFICATION)

Replicates	H_2O Control	Liquid Ingredients
1	0.760	0.745 (0.02*)
2	0.768	0.830 (−0.08)
Average	0.764	0.788 (−0.05)

S.D.$_3$ = 0.090; df = 13. Used to test all treatment differences.

the reason for failing to show statistical significance when the inference is to be drawn for all teeth, instead of just for the type and size of teeth used in the two reps, is the low power of the test. The estimate of the error mean square is based on only one degree of freedom. This is because the experiment involving two treatment groups is replicated only two times. With more replications, a statistically significant difference would probably be attained. The error term used in testing for treatment differences is significantly larger than basic error. This is probably due to a difference in protection by the paste on different sizes and/or types of teeth. (*c*) In the three replications of the paste without petrolatum run against water controls, there was very little decalcification in the former (Table 4). Note that the amount of decalcification occurring when the paste is used is essentially a constant for all reps. Because of this essentially constant decalcification when the paste is used and the large change in amount of decalcification from one rep to another when H_2O is used, the standard over-all test for treatment differences does not show significance. However, testing treatment differences for each run separately, decalcification is significantly decreased, by using the paste, for all replications. Since past chemical histories of the various control teeth probably vary considerably, this may explain the situations wherein controls and the paste without petrolatum may be essentially the same; hence the described testing does

TABLE 3

EFFECTIVENESS OF VARIOUS SnF_2 PROPHYLAXIS PASTE COMPONENTS (MG. PER CENT PHOSPHORUS LOST DURING DECALCIFICATION)

Replicates	H_2O Control	Complete Formula except Silicone
1	0.895	0.125 (86.0*)
2	1.202	0.095 (92.1)
Average	1.049	0.110 (89.0)

$S.D._1 = 0.337$; $df = 1$. Used to test average treatment differences.

$S.D._2 = 0.083$; $df = 12$. Used to test treatment differences for each replicate separately.

TABLE 4

EFFECTIVENESS OF VARIOUS SnF_2 PROPHYLAXIS PASTE COMPONENTS (MG. PER CENT PHOSPHORUS LOST DURING DECALCIFICATION)

Replicates	H_2O Control	Complete Formula except Petrolatum
1	0.518	0.032 (93.8*)
2	0.895	0.062 (93.1)
3	1.202	0.030 (97.5)
Average	0.872	0.042 (94.8)

$S.D._1 = 0.486$; $df = 2$. Used to test average treatment differences.

$S.D._2 = 0.073$; $df = 17$. Used to test treatment differences for each replicate separately.

not allow one to draw the inference that the paste is effective for all sizes and types of teeth or of varied derivation. However, it gives a strong indication that this may be so. (*d*) In the three replicate studies where stannous fluoride was omitted from the paste formulation, the protection without the stannous fluoride was not significantly different from that of the water controls (Table 5). (*e*) When silex was omitted from the paste, the protection afforded by the paste was significantly better than the water controls ($P>0.05$) (Table 6). This estimate of the standard deviation used for testing this effect is based on only one degree of freedom.

Phase III.—Averaged over all five reps, the 1:7 proportion of stannous fluoride to silex differed from the 1:1, 3:5, and 1:3 proportions in effectiveness of the paste ($P>0.05$), and the water controls differed from all four paste treatments at the 1 per cent level of significance. (Table 7). A linear decreasing trend on the average effectiveness of the paste (averaged over reps) with a decrease in the proportion of stannous fluoride to silex was significant at the 5 per cent level.

Phase IV.—Weekly testing for 5 weeks was accomplished, using the same batch of paste, but different teeth each week. A control group (H_2O) was also included each week. Therefore, it appears that there is negligible decalcification of teeth in this study which were treated with the paste.

TABLE 5

EFFECTIVENESS OF VARIOUS SnF_2 PROPHYLAXIS PASTE COMPONENTS (MG. PER CENT PHOSPHORUS LOST DURING DECALCIFICATION)

Replicates	H_2O Control	Complete Formula except SnF_2
1	0.648	0.658 (−0.02*)
2	1.138	1.165 (−0.02)
3	1.130	1.150 (−0.02)
Average	0.972	0.991 (−0.02)

S.D.$_3$ = 0.043; df = 20. Used to test all treatment differences.

TABLE 6

EFFECTIVENESS OF VARIOUS SnF_2 PROPHYLAXIS PASTE COMPONENTS (MG. PER CENT PHOSPHORUS LOST DURING DECALCIFICATION)

Replicates	H_2O Control	Complete Formula except Silex
1	1.048	0.342 (67.4*)
2	0.760	0.155 (79.6)
Average	0.904	0.249 (73.5)

S.D.$_3$ = 0.248; df = 13. Used to test all treatment differences.

DISCUSSION

The stannous fluoride, silex, silicone prophylaxis paste gave a greater protective effect to teeth subjected to the in vitro decalcification procedures than did a single topical application of a 10 per cent stannous fluoride solution. The increase in effectiveness is possibly due to a series of concurrent and synergistic reactions. When the prophylaxis paste is used, the placement of stannous fluoride on the tooth structure and the cleaning action of the silex are simultaneous; the possibility of saliva contaminating the field is thus eliminated, and a maximum anticariogenic effect is insured.

Silex appears to potentiate the protective effect of the stannous fluoride. The use of liquid petrolatum as the moistening agent increases the effectiveness of the mix. Results are rather erratic, however, and the use of a 1:5 silicone-petrolatum solution with the silex and stannous fluoride affords a constant high protection to in vitro decalcification. Since the silicone-petrolatum fluid alone gives little or no protection, possibly the synergistic silicone stannous fluoride action is due to the fact that the tin, plus the contact temperature generated by the rapidly rotating rubber cup, acts to catalyze the polymerization of the silicone fluid.

TABLE 7

EFFECT OF VARYING PROPORTIONS OF STANNOUS FLUORIDE TO SILEX (MG. PER CENT PHOSPHORUS LOST DURING DECALCIFICATION)

Replicates	H_2O Control	1:1	3:5	1:3	1:7
1	1.055	0.028 (97.3*)	0.242 (77.1*)	0.330 (68.7*)	0.810 (23.2*)
2	1.078	0.260 (75.8)	0.258 (76.1)	0.548 (49.2)	0.742 (31.2)
3	1.118	0.355 (68.2)	0.608 (45.6)	0.842 (24.7)	0.705 (36.9)
4	1.120	0.468 (58.2)	0.018 (98.4)	0.130 (88.4)	0.425 (62.0)
5	1.195	0.005 (99.6)	0.010 (99.2)	0.000 (100.0)	0.578 (51.6)
Average	1.113	0.223 (79.8)	0.227 (79.2)	0.370 (66.2)	0.652 (41.0)

$S.D._1 = 0.372$; $df = 16$. Used to test average treatment differences.
$S.D._2 = 0.224$; $df = 72$. Used to test treatment differences for each replicate separately.

TABLE 8

EFFECTIVENESS OF SnF_2 PROPHYLAXIS PASTE AS FUNCTION OF TIME (MG. PER CENT PHOSPHORUS LOST DURING DECALCIFICATION)

Time (Days)	H_2O Control	SnF_2 Paste
0	1.145	0.105 (90.8*)
1	1.202	0.010 (99.2)
5	0.760	0.040 (94.7)
8	1.028	0.022 (97.8)
32	1.195	0.002 (99.8)
Average	1.066	0.036 (96.5)

$S.D._4 = 0.058$; $df = 14$. Used to test time difference in paste.
$S.D._5 = 0.067$; $df = 15$. Used to test time difference in H_2O controls.

The effectiveness of the paste appears to be linearly related to the ratio of stannous fluoride to silex, at least for the range 1:1–1:7. A decrease in the concentration of stannous fluoride causes a decrease in the protective effect of the paste.

This 24-hour decalcification test is severe, and the pH developed (pH 2.8) is far below that encountered under oral physiological conditions. Thus the use of the paste produces a tooth surface that is resistant to acid decalcification for a longer period of time than has been observed after the topical application of 10 per cent SnF_2 solution.

Until proved otherwise, any future use of this paste should not obviate the requirement for a topical application of a 10 per cent solution of stannous fluoride. Instead, it is intended to be used in the cleaning and polishing phase of the phophylactic treatment as another method of decreasing the solubility of the tooth surface. Thus, with no additional time or manpower requirements, the benefits derived from the use of the stannous fluoride, silex, silicone dental prophylaxis paste can augment those of the subsequent stannous fluoride application and can thus maximize the protective effect.

SUMMARY

A stannous fluoride, silex, silicone dental prophylaxis paste has been formulated that more effectively inhibited in vitro solubility of phosphorus of tooth structure than did a topical application of 10 per cent stannous fluoride. The silex, which acts as the abrasive, did not react with any of the other ingredients. The silicone-petrolatum moistening agent increased the protective effect and added stability to the mixture. In a time-function study the effectiveness of the paste did not decrease over a 5-week period. Stannous fluoride was the salient constituent of the dental prophylaxis paste. The other components of the mixture either added more desirable properties or increased its effectiveness.*

The authors are grateful for the statistical support of Richard C. McNee, Department of Biometrics, and for technical assistance by A2C Ernest Herrera and A2C Judith Martin.

REFERENCES

1. Bibby, B. G., Zander, H. A., McKelleget, M., and Labunsky, B. Preliminary Reports on the Effect on Dental Caries of the Use of Sodium Fluoride in a Prophylactic Cleaning Mixture and in a Mouthwash, *J. D. Res.*, **25**:207, 1946.
2. Bibby, B. G. Fluoride Mouthwashes, Fluoride Dentifrices and Other Uses of Fluorides in Control of Caries, *J. D. Res.*, **27**:367, 1948.
3. Harris, N. O., and Hester, W. R. *The Effectiveness of a Topical Application of Stannous Fluoride in a Preventive Dentistry Program: A Review of the Literature,* School of Aviation Medicine, USAF, Review 2-59, September, 1958.
4. Walsh, J. P., and Green, R. W. The Influence of Some Surface-active Substances on Decalcification of the Enamel Surface, *J. D. Res.*, **29**:270, 1950.
5. Taussky, H. H., and Shorr, E. J. A Microcolorimetric Method for the Determination of Inorganic P., *J. Urol.*, **69**:454, 1953.
6. Phatak, N. M., Savara, B. S., and Dement, J. A. New Silicone in Dental Practice (Preliminary Report), *J. D. Res.*, **34**:788, 1955.
7. Buonocore, M. G., Evaluation of Resin Film-forming Materials for Use as Bonding Agents between Resin Filling Materials and Tooth Structure, *J. D. Res.*, **34**:749, 1955.
8. Savara, B. S., Phatak, N. M., and Dement, J. A. In Vitro Study of Change in Contact Angle of Water on Tooth Surface Induced by Different Preservatives and Silicone, *J. D. Res.*, **37**:24, 1958.

* In order to attain patient taste acceptance and to eliminate the limited number of adverse gingival reactions, the formula of the prototype paste was altered to include a lowered stannous fluoride content and the addition of sodium borate as a taste-masking agent. The formula used is as follows: stannous fluoride, 100 gm.; silex, 300 gm.; sodium borate, 30 gm.; sucaryl, 3.0 gm.; silicone, 20 ml.; liquid petrolatum, 80 ml.; oil of peppermint, 5 ml.; and oil of anise, 5 ml.

Vascular Anastomoses in the Region of the Mandibular Symphysis

DAVID R. REDDEN, JACK G. BISHOP, JAMES L. MATTHEWS, and HOMER L. DORMAN

Department of Physiology, Baylor University College of Dentistry, Dallas, Texas

Unilateral and bilateral mandibular resection and the treatment of compound fractures of the mandible indicate that the mandibular artery may be ligated without compromising the vitality of the tissues distal to the ligature.[1–3] The exact pattern of the circulatory complex in this tissue are is not sufficiently understood to explain these findings. Hawkins[3] has suggested that the vascular anastomosis across the mid-line of the mandible is limited but that the lips and the neighboring soft tissue contain many anastomotic branches of the mental and facial arteries. Ryder[4] found that the canals and foramina of the mandible transmit anastomosing vessels which connect the inferior dental and sublingual arteries. MacGregor[5] cannulated both mandibular arteries of the fractured jaw of a post mortem subject and reported that an anastomosis existed across the symphysis. Recently Bishop, Matthews, Dorman, and Moore[6] injected thorium dioxide into the mandibular artery of dogs. Radiographic analysis revealed a minimum amount of crossover between the left and right side of the mandible with a minimum perfusion pressure.

A study of the blood pressure in the cannulated mandibular arteries of dogs was therefore undertaken in an attempt to resolve these viewpoints.

EXPERIMENTAL METHODS

Mongrel dogs of varying age and sex were anesthetized with sodium pentobarbital, 33 mg/kg body weight. The blood of each animal was rendered non-coagulable by intravenous injection of heparin, 5 mg/kg, 30 minutes before each experimental series was undertaken. Blood pressure was recorded by use of a calibrated strain gauge,* amplifier,† and ink-writing oscillograph.

The right mandibular artery was exposed by an inframandibular incision lateral to the mid-line. The right diagastric muscle was dissected free of deep fascia and pulled laterally by a ligature around the belly of the muscle. Dissection was continued until the internal pterygoid muscle and its insertion were separated from the mandible. The exposed mandibular artery was tied off near the internal maxillary artery, and a polyethylene cannula which had a bore size that approximated the lumen size of the artery was inserted distally.

This study was supported by Research Grants D-318 and, in part, DT-21 from the N.I.D.R., U.S. Public Health Service.

Received for publication May 6, 1960.

* Statham Instruments, Los Angeles, Calif.

† Brush Electronics Co., Cleveland, Ohio.

The right femoral artery was ligated distal to the inguinal ligament and cannulated. The mandibular cannula was connected to the junction of a 10-ml. syringe (Fig. 1) and to the pressure transducer. Back-pressure in the mandibular artery was continuously monitored, while removal of the femoral cannula clamp allowed intermittent measurement of the systemic blood pressure. The syringe was used to inject thorium dioxide, and the cannula arrangement permitted the perfusion pressure to be observed.

Appropriate incisions were used to expose the left and right common carotid arteries, and heavy cord ligatures were used to occlude these vessels for short periods. Mechanical blocks of the mandibular symphysis were accomplished by

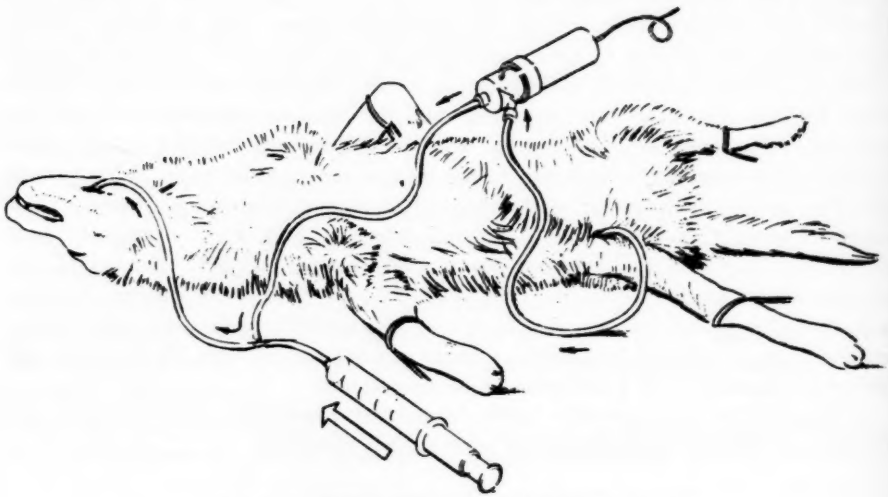


FIG. 1.—Cannula connections in the perfused animal

means of a hemostat clamped so as to include the skin and the lingual aspect of the gingiva. Radiographs were made, using a dental X-ray unit on fine-grain no-filter film.

RESULTS

Measurements of femoral blood pressure were made as soon as the cannula system was in place and the values obtained averaged 175 mm. of mercury. Following these determinations the femoral cannula was clamped, and the pressure in the cannulated distal segment of the right mandibular artery was recorded. Back-pressures in the right mandibular artery (Table 1) ranged between 100 and 135 mm. of mercury with a mean of 115 mm. If the femoral blood pressure can be taken as an index to the pressure in the proximal segment of the mandibular artery, these values represented a back-pressure which was only 30 per cent less than the normal forward-perfusing pressure of the blood column. Obviously, then, arterial blood entered the distal branches of the mandibular artery from sources other than the proximal branch from the internal maxillary artery.

Occlusion of the left common carotid artery while measuring back-pressure in

the right mandibular artery resulted in a decrease in back-pressure from an average of 115 to 90 mm/Hg, while occlusion of the right common carotid artery decreased the back-pressure to 103 mm/Hg (average). Occlusion of the left and right common carotid arteries decreased the back-pressure in the right mandibular artery to an average of 68 mm/Hg (Fig. 2). A mechanical clamp across the mandibular symphysis between the lingual aspect of the gingiva and the skin surface reduced the back-pressure to 78 mm/Hg. Back-pressure in the distal portion of the right mandibular artery was reduced to 53 mm/Hg, but not eliminated, by clamps on both left and right common carotid arteries and a mechanical block on the mandibular symphysis applied simultaneously (Fig. 3). Insertion of the finger into the mouth and application of substantial pressure to the tissues overlying the left mandibular canal reduced the back-pressure in the right mandibular artery to 53 mm/Hg (Fig. 4). The back-pressure decreases resulting from these manipulations are summarized in Figure 5.

TABLE 1

AVERAGE SYSTEMIC AND BACK-PRESSURE DATA WITH BLOOD SUPPLY MANIPULATION

Animal No.	Femoral Pressure	Back-Pressure in Mandibular Artery	Back-Pressure in Right Mandibular Artery with				
			Right Carotid Block	Left Carotid Block	Left and Right Carotid Block	Symphysis Block	Symphysis and Carotids
1	175	100	84	77	63	.....	.....
2	160	100	88	76	50	75	.....
3	180	125	60	57	50	75	.....
4	170	117	.....	130	95	.....	.....
5	170	135	115	85	75	60	.....
6	175	100	95	85	50	55	40
7	185	120	117	88	68	75	50
8	160	115	115	105	85	90	65
9	175	122	115	95	65	85	50
10	185	130	125	100	85	103	66
11	190	130	122	92	63	85	47
Mean	175	117.2	103.6	90	68	78.1	53

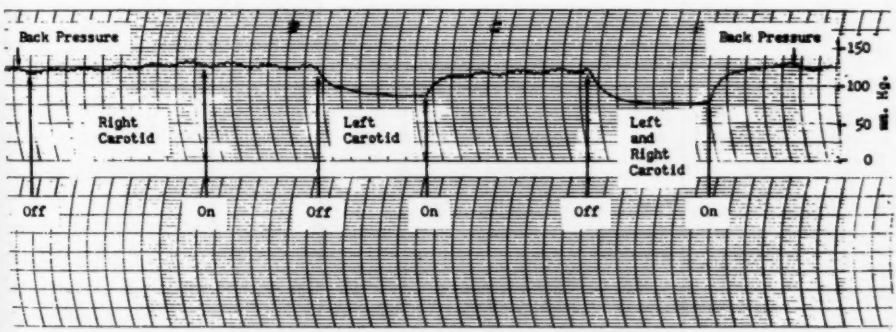


FIG. 2.—Record of effect of blood supply manipulation on back-pressure

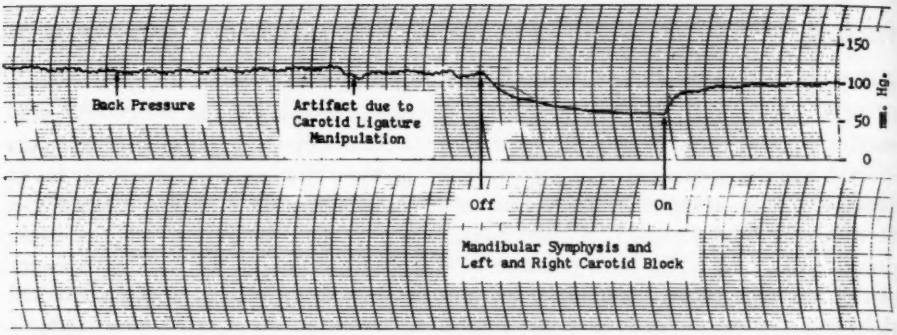


FIG. 3.—Record of back-pressure drop due to mechanical block of the mandibular symphysis and left and right carotid arteries.

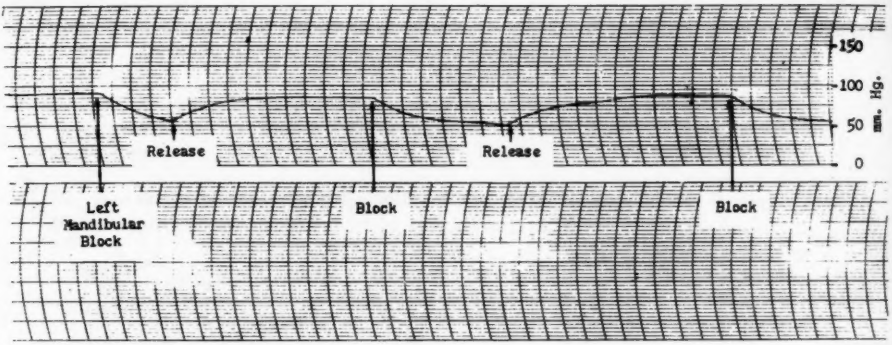


FIG. 4.—Back-pressure drop in the right mandibular artery with pressure block on the left mandibular artery.

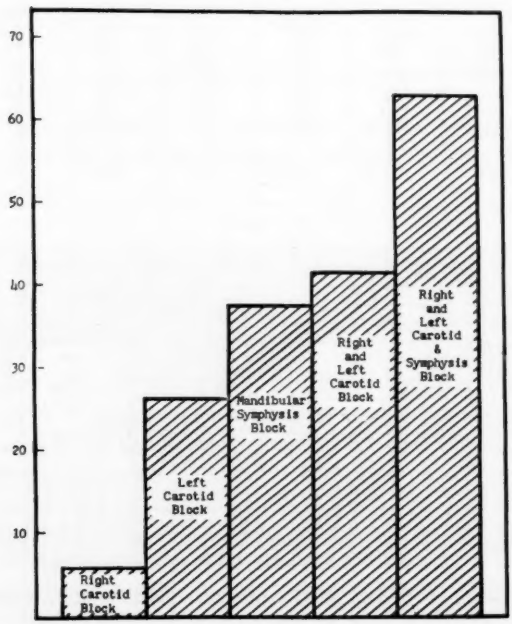


FIG. 5.—Average back-pressure drop with common carotid artery and mandibular symphysis block

Further evidence that the back-pressure drops obtained by occlusion of several blood vessels represent functional anastomotic pathways was obtained from radiographic observations during perfusion of the left mandibular artery at various known perfusion pressures. Perfusion at 100 mm/Hg pressure for 70 seconds of thorium dioxide into the left mandibular artery was sufficient to visualize radiographically the right mandibular artery, its venous drainage, and the mental branch (Fig. 6). There was no evidence of crossing over at this perfusion pressure. During perfusion pressure of 115 mm/Hg (Fig. 7), thorium dioxide entered the symphysis and passed across into at least two vessels which entered the symphysis from the floor of the mouth. Perfusion at a pressure of 180 mm/Hg (Fig. 8) allowed the thorium dioxide to pass from the right mandibular artery and fill vessels located along the labial side of the left mandible. Perfusion at 200 mm/Hg allowed the thorium dioxide to fill the left mandibular artery and its mental branch as well as all the vessels which were visualized at the lower perfusion pressures (Fig. 9).

DISCUSSION

The back-pressure data on 11 animals consistently indicated the presence of anastomosis between the left and right mandibular artery or collateral circulation pathways which joined the mandibular artery. The data indicated that if collateral circulation alone was responsible for the consistent back-pressure, the collateral vessel or vessels must anastomose with the mandibular artery on both sides of the mandible. This indication was first seen when the left common carotid was ligated and was followed by a subsequent drop in back-pressure in the right mandibular artery.

Further information which led to the definite indication of anastomosis across the mandibular symphysis was obtained by placing a pressure block on the left mandibular artery at the mandibular foramen while recording pressure from the right mandibular artery.

The radiographs show an anastomosis between the left and right mandibular arteries, as well as other anastomoses with the branches of the lingual and several other arteries which enter the mandible at or near the mandibular symphysis.

These data indicate that a complex of anastomotic pathways exists in the area of the mandibular symphysis in dogs. All attempts to reduce this existing back-pressure to zero by manipulation of the main arterial supplies to the area failed to produce a drop lower than an average of 53.0 mm/Hg. A drop of the back-pressure to zero was accomplished only by obstruction of the ascending aorta. Failure of the back-pressure to drop to zero following blockage of both common carotids may be explained on the basis of pressures transmitted through the vertebral arteries across the Circle of Willis and thence into the maxillary and mandibular arteries.

The absence of crossover during perfusion of thorium dioxide at 100 mm/Hg is difficult to interpret without assuming that this compound inflicts some functional insult to the tissues which it contacts. Nevertheless, the data indicate that the pressure gradient necessary to permit crossover between the left and right mandibular arteries is higher than that required to induce crossover from the right mandibular artery into the anastomotic branches of the sublingual vessels.

The functional complexity of the anastomotic connections which occur at the

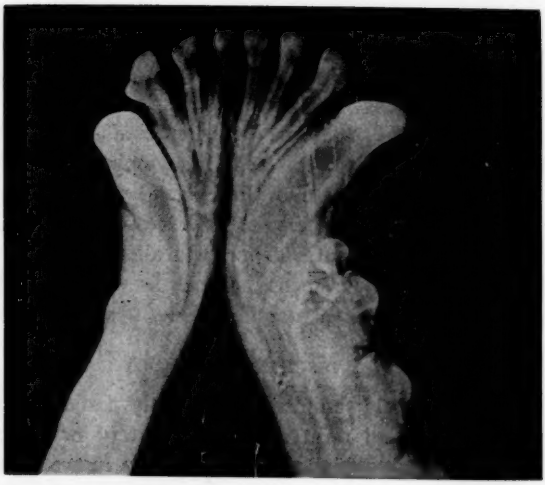


FIG. 6.—Radiograph of thorium dioxide infusion into the right mandibular artery at 100 mm/Hg

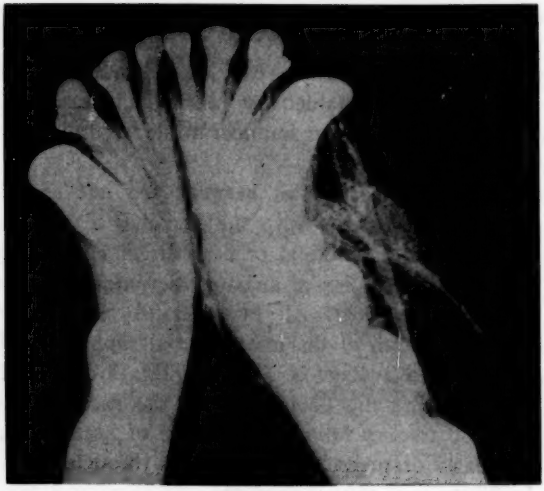


FIG. 7.—Radiograph of thorium dioxide infusion into the right mandibular artery at 115 mm/Hg

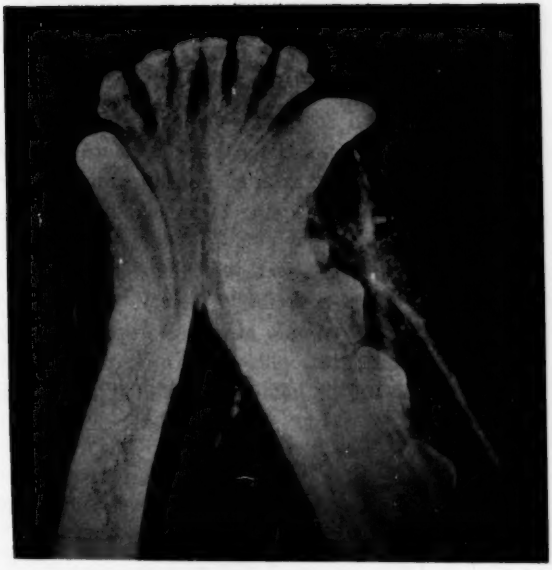


FIG. 8.—Radiograph of thorium dioxide infusion into the right mandibular artery at 180 mm/Hg

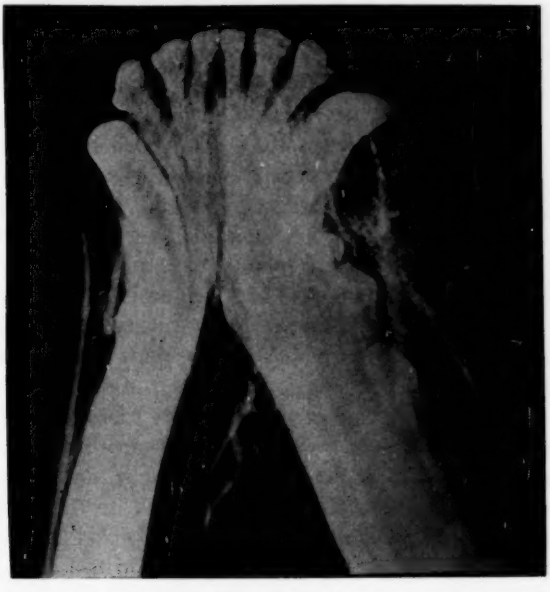


FIG. 9.—Radiograph of thorium dioxide infusion into the right mandibular artery at 200 mm/Hg

mandibular symphysis offers an explanation for the continued vitality of tissues distal to severed, ligated, or functionally obstructed mandibular arteries.

SUMMARY

The back-pressure in the distal segment of the ligated right mandibular artery of dogs was found to average 115 mm/Hg. A back-pressure of 53 mm/Hg remained after clamping both common carotid arteries and the mandibular symphysis, and zero back-pressure could be obtained only after clamping the ascending branch of the aorta.

Numerous anastomotic blood vessels were visualized radiographically by infusion of thorium dioxide into the right mandibular artery. Crossover between the left and right mandibular arteries occurred only at perfusion pressures above 100 mm/Hg.

The presence of this anastomotic complex offers an explanation for the continued vitality of tissues distal to severed or ligated mandibular arteries following fracture or mandibular resection.

REFERENCES

1. Euler, H. The Blood Supply to the Mandible and Its Teeth after Ligation of the Inferior Dental Artery, *Deut. Monatsschr. Zahnheilk.*, **40**:193, 1922.
2. Perint, J. Roentgenologic Examination of the Blood Supply in the Jaws and Teeth by Applying Radiopaque Solutions, *Oral Surg., Oral Med., Oral Path.*, **2**:2, 1949.
3. Hawkins, C. H. Blood Supply to the Lower Jaw, *Proc. Roy. Soc. Med.*, **29**:506, 1935.
4. Ryder, W. B. Significance of Circulatory Canals in Roentgenograms of the Alveolar Process, *Am. J. Orthodont. Oral Surg.*, **28**:640, 1942.
5. MacGregor, A. Arterial Supply to the Mandible, *Proc. Roy. Soc. Med.*, **39**:347, 1946.
6. Bishop, J. G., Matthews, J. L., Dorman, H. L., and Moore, E. E. Blood Flow and Blood Pressure in the Mandibular Artery, *J. D. Res.*, **38**:244, 1959.

An Investigation of Enamel Thickness in Human Lower Incisor Teeth

BARRIE GILLINGS and MICHAEL BUONOCORE
Eastman Dental Dispensary, Rochester, New York

The thickness of enamel in human teeth has received little attention from dental research workers other than occasional mention of measurements on specific areas. The standard texts on dental anatomy make no reference to enamel thickness beyond the statement that the enamel is thickest at the incisal or occlusal surfaces and decreases in thickness gingivally.[1–10] Opinions concerning the actual and comparative thickness of the enamel covering the buccal, lingual, mesial, and distal surfaces are unsupported by published data. Questions bearing on other morphologic characteristics, such as the relationship between enamel thickness and crown size, have not been adequately answered. In order to fill part of this gap in our knowledge of dental anatomy, the following work was undertaken.

MATERIALS AND METHODS

In order to measure the thickness of enamel in a tooth, we must first cut it to expose the enamel. The shortest distance from a point on the enamel surface to the underlying dentinoenamel junction could then be determined, and this would be a radial enamel thickness measurement at this point. This measurement may vary according to the section plane adopted, even though made from the same point on the external enamel surface, such as the middle of the buccal surface illustrated in Figure 1, since either the longitudinal or the transverse section plane, or both, could cut the enamel obliquely at the point where the measurement of thickness is made because of the curvature of the enamel surface. A measurement made on enamel exposed by such cuts would not indicate the true radial enamel thickness. A measurement incorporating an error resulting from an oblique cut we term an "apparent radial enamel thickness." In order to obtain a true radial enamel thickness measurement from a given section, we must therefore consider the curvature of the surface in both the incisogingival and the mesiodistal directions.

Since the purpose of this study was to determine the enamel thickness on all four surfaces of lower incisor teeth, the transverse plane of section was adopted. Although this plane is likely to produce errors in measurements made directly on the sections for the reasons already discussed, we can compensate for these errors by the use of a graphical reconstruction, which allows true radial enamel thickness measurements to be made at any point on the tooth surface. The technique and rationale for this procedure are described below by analogy to a loaf of bread.

If one were to cut a loaf of bread transversely into thin slices, it would be possible

This study was supported in part by Research Grant D-536 from the N.I.H., U.S. Public Health Service, and the Colgate-Palmolive Company.

Received for publication May 10, 1960; revised by authors July 14, 1960.

to reassemble the slices so that they resembled the original loaf (see Fig. 2, *A*). Similarly, it should be possible to reassemble sections cut from a tooth, to resemble the original tooth (see Fig. 3). Now let us suppose that the cut loaf has been reassembled and now is cut again, longitudinally, down the middle, as indicated by the shaded area in Figure 2, *A*. If we separate the two halves produced by this second cut and examine the surface of one of the halves, we will see a rectangular shape made up by the base of the loaf, the outer surfaces of the two end slices, and the curved top (longitudinal section plane in Fig. 2, *A*). This surface will show vertical parallel lines, which are the transverse cuts made originally. It is apparent that, by taking

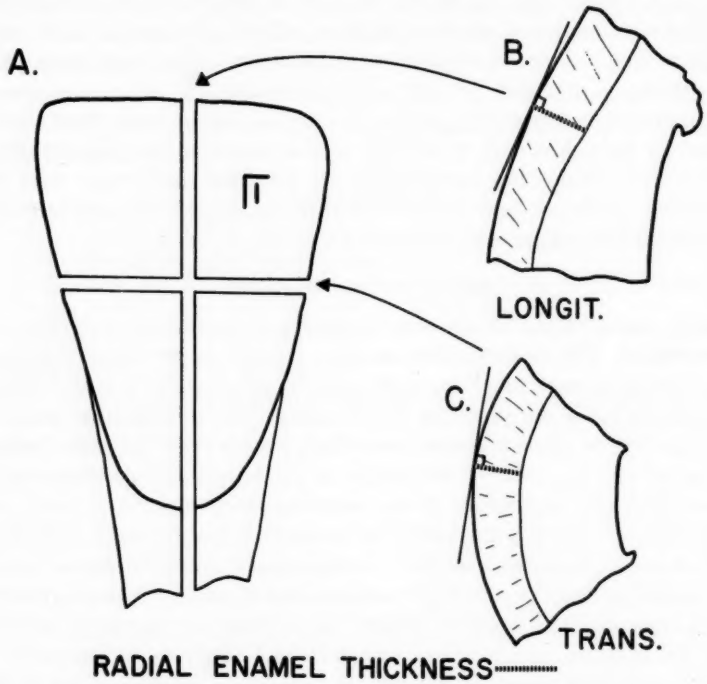


FIG. 1.—Radial enamel thickness. (*A*) Two cuts, exposing enamel in the middle of the buccal surface of a lower incisor. (*B*) Longitudinal section. (*C*) Transverse section. Radial enamel thickness measurements are not necessarily the same when measured on *B* and *C*.

appropriate measurements on the longitudinal section plane, the outline could be recorded on co-ordinate paper. This is a simple procedure with a large object like a loaf of bread but would be a difficult one with a small object.

Actually, the longitudinal cut is not necessary to determine the longitudinal outline of the loaf, since we can draw this outline by using direct measurements of the original transverse slices. The top and bottom surfaces of the loaf could be marked with lines corresponding to where this longitudinal section would have cut these surfaces. The points where the longitudinal lines and original transverse cuts meet could be labeled in order 1_I, 1_{II}, 2_I, 2_{II}, etc., as in Figure 2, *A*. We could now draw

on co-ordinate paper a line to represent the base of the loaf and draw perpendicular to this line a series of perpendicular, parallel, evenly spaced lines corresponding to the separation of our original transverse slices.

By laying the base of each transverse slice, in turn, on its corresponding graph line, with points 1_1, 2_1, 3_1, etc., on the base line, and transposing the points 1_{11}, 2_{11}, 3_{11}, etc., onto the vertical lines, we are left with a series of points, which, when joined up with a smooth line from 0_{11} *to* 10_{11}, record on the co-ordinate paper the outline of the longitudinal section which would have resulted, had we not merely marked the longitudinal plane but actually cut along it (see Fig. 2, *B*).

Further, if the plane of longitudinal section passed through, say, a large void in the middle of the loaf, this void would appear in some of the transverse sections, and

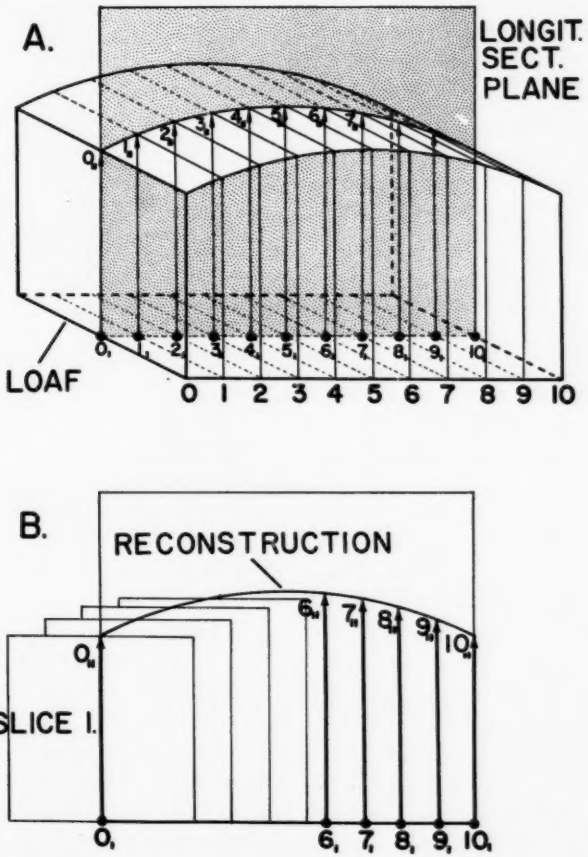


FIG. 2.—Graphical reconstruction technique. (*A*) Loaf of bread cut into ten transverse slices, 1, 2, 3, etc. An imaginary longitudinal section plane (*shaded area*) intersects the bottom and top surfaces of the slices at 0_1, 0_{11}, 1_1, 1_{11}, 2_1, 2_{11}, etc. (*B*) Graphical reconstruction of the longitudinal section plane shown in *A*. Slices *1–4* are shown lying in the plane of the paper, with 0_1 and 0_{11}, 6_1 and 6_{11}, etc., transferred to the reconstruction drawing.

its position and shape in this longitudinal plane could be recorded by measurements and transposition to the co-ordinate paper in the manner just described. Other planes of section, in any direction, flat, curved, or skewed, could be drawn from the transverse sections using the same technique, without actually cutting the loaf in these planes.

In cutting a tooth into thin sections there is an unavoidable loss of material due to the thickness of the blade cut, and we must accordingly make allowance for this loss. If we space the individual sections according to the amount lost, we can reproduce the original spatial configuration of the tooth.

Now suppose a tooth in the middle of a rectangular plastic block is sectioned serially in the transverse plane. We can determine what any desired longitudinal section of the tooth would have looked like, by drawing on co-ordinate paper a series of correctly spaced parallel lines and marking on these lines the spatial positions occupied by the points where our desired axial section plane intersects the sections making up our reassembled tooth. This would be difficult, using the actual sections. Enlarged tracings on co-ordinate paper of the actual sections and surrounding square or rectangular plastic supports make it simple, and, furthermore, we can reproduce planes of section which would be impossible to duplicate by any actual cutting process. We call this technique "graphical reconstruction."

In order to use this graphical reconstruction technique in a tooth sectioned as described above, for the measurement of enamel thickness, the following conditions must be met: (1) the plane of section should, as nearly as possible, be perpendicular to the long axis of the tooth; (2) the tooth must be mounted in a block of such a shape that the cut sections can be reassembled in their original spatial positions; (3) each section cut must be of uniform thickness; (4) the amount of material removed by the blade must be uniform for each section cut; and (5) sections should be marked so that the order in which they were cut can be identified.

In this study, the above conditions were met by mounting the teeth to be sectioned in rectangular blocks of plastic.* The blocks were provided with a flat base and a straight edge, with the long axis of the tooth lying parallel to the base. These blocks were then mounted on the cutting table of a section machine† so that the section plane was at right angles to the long axis of the tooth. This procedure fulfils conditions 1 and 2. The technique and apparatus used for cutting has been described in a previous paper[11] and resulted in the production of semiserial sections of 120 μ thickness, made at 450-μ intervals. Since the machine made cuts of uniform thickness, conditions 3 and 4 are fulfilled, and the spatial position of each section could be deduced from a knowledge of the constant distance the blade was moved in relation to the specimen to produce each section. The section thickness and the width of the blade cut together equal this distance (Fig. 3). Before cutting, each plastic block was marked with three grooves, as illustrated in Figure 4, *A*. The orientation and order in which the sections were cut were determined on the basis of the lateral shift of the single groove in the plastic block (see Fig. 4, *B*), thus fulfilling condition 5. The double grooves indicated in the diagram serve to identify one side of the block, to

* Ward's Bioplastic, Ward's Natural Science Establishment, P.O. Box 1712, Rochester 3, New York.

† Bronwill Gillings Thin Section Machine, Will Corporation, 39 Russell Street, Rochester, New York.

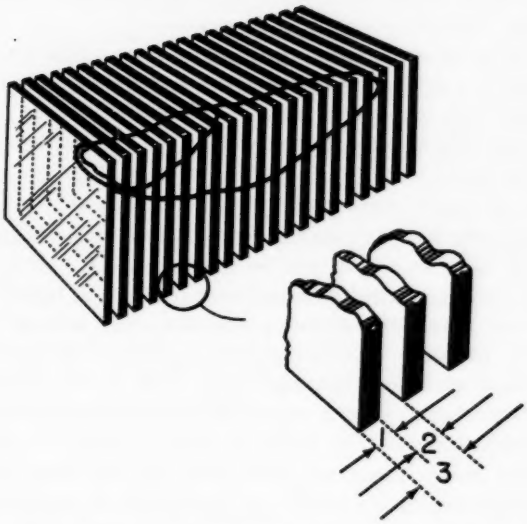


FIG. 3.—Reassembled tranverse sections of a tooth in a plastic block. Insert shows *1*, section thickness; *2*, blade cut; *3*, cutting interval (none to scale). The section thickness and the width of cut made by the blade together equal the cutting interval.

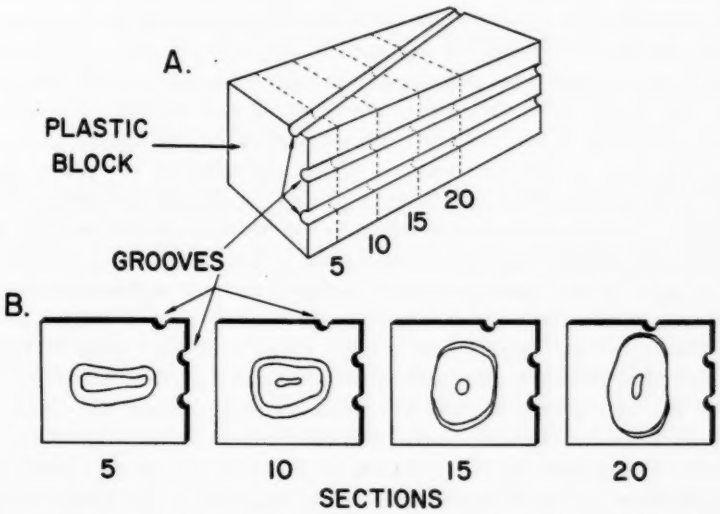


FIG. 4.—Method used to establish section sequence. (*A*) Plastic block containing a tooth and marked with three reference grooves. (*B*) Sections *5*, *10*, *15*, and *20* from *A*, showing how the change in position of the single groove can be used to establish the section sequence.

prevent misplacement of a section because of its being viewed from the wrong side, which would change the position of its single groove.

Cut sections were mounted, in order, on 3″ × 4″ glass slides with Canada balsam, enlarged 25.4 times in a projecting microscope,* and traced onto 1-inch co-ordinate paper, using the flat base and square edge of the plastic support of each section as reference co-ordinates. Each tooth studied yielded, on the average, 23 such tracings for the enamel-containing part of the tooth. The tracings of the projected images were always made from the upper surface of the sections, thus eliminating tracing errors due to surface slope in each actual section. Since measurements of these tracings were made to the nearest 0.050 inch, they can be assumed to have an accuracy of $\pm 25\mu$.

For this study, 27 intact, caries-free adult lower central and lateral incisors showing minimum incisal wear were selected from a pooled sample, without regard to size or age, and imbedded in plastic as described above. It was felt that these two tooth types showed less individual variation than any other teeth of the human dentition and could therefore be expected to show a pattern of enamel distribution and morphology which would be closely similar from tooth to tooth, if such a pattern existed. The investigator felt that he could not separate lower centrals from lower laterals in a pooled sample with any degree of certainty, so both centrals and laterals were investigated under the common heading of lower incisors.†

On each tracing of each section of a particular tooth, points were located on the outer enamel surface to designate areas of a representative enamel thickness for the buccal, lingual, mesial, and distal surfaces, respectively, from which measurements were to be made. (The term "representative enamel thickness" is used to designate the areas considered to be of average thickness for a particular surface, but, to avoid confusion, the word "average" is not used in this context, since it is used in this paper when measurements are combined to give an "average" enamel thickness measurement.) In Figure 5, *B*, such points are shown in four sections of a typical series, where the solid lines bisect the outer enamel surface on the buccal, lingual, mesial, and distal surfaces of the four sections. Using the edges of the plastic support as reference co-ordinates, these points were plotted on co-ordinate paper, on parallel lines spaced at a scale distance equivalent to the distance separating each transverse section from its neighbor. Lines connecting the points so obtained from all the sections of a given tooth crown produce an outline representing the appearance of the outer surface of the tooth which would have resulted from a longitudinal section through the buccal and lingual outer enamel surfaces of the crown, in Figure 5, *A*. (An outline obtained in the same way in the mesiodistal plane is illustrated in Fig. 5, *C*.) Since the longitudinal section was made to pass through the areas of representative thickness on both the buccal and the lingual surface, it may be curved or skewed. (This is evident from the location of points on the outer enamel surface of the example shown in the sections illustrated in Fig. 5, *B*.) The radial enamel thick-

* Bausch and Lomb Tri-Simplex Micro-Projector.

† This conclusion was arrived at after testing the performance of fifteen dentists in arranging twenty-seven randomly selected lower incisor teeth into two groups, centrals and laterals. None of the fifteen made the same selection, and total agreement was reached on only four of the twenty-seven teeth. Further, not one of the fifteen duplicated his previous selection, on repeat testing with the same group of teeth.

ness was now measured for each representative area in each section and plotted on the same drawing. This radial thickness is the shortest distance from the points designating the areas of representative thickness on the buccal, lingual, mesial, and distal surfaces to the underlying dentinoenamel junction. Lines connecting the points so obtained represent the outline of the cut dentinoenamel junction if a longitudinal cut had been made to pass not only through the areas of representative thickness but also through these areas in a radial direction, at each section level (see Fig. 5, *A* and *C*). The pulp-chamber outline has also been recorded in a similar fashion.

It should be realized that although the external enamel surface is correctly rep-

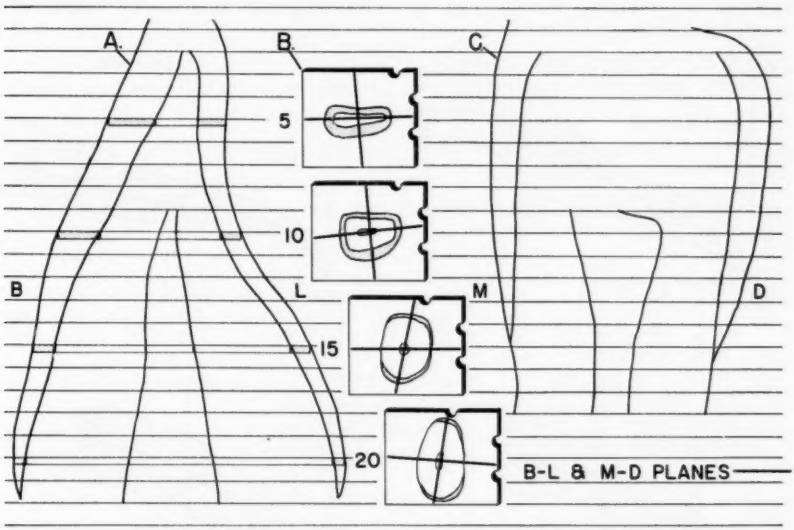


FIG. 5.—Typical graphical reconstruction of the buccolingual and mesiodistal section planes in a lower incisor tooth. (*A*) Buccolingual graphical reconstruction, with sections *5, 10, 15,* and *20* superimposed. (*B*) Sections *5, 10, 15,* and *20* of the series of sections used to construct *A* and *C* (not to scale with *A* and *C*). The areas of representative enamel thickness for the four surfaces can be located where the buccolingual and mesiodistal section planes intersect the outer enamel surface. (*C*) Mesiodistal graphical reconstruction.

resented in Figure 5, *A* and *C*, and conveys a true outline of what the tooth would have looked like, had it been cut in the longitudinal plane, the radial distance between the outer enamel surface and the dentinoenamel junction as plotted is actually shorter than would be obtained from direct measurements on a longitudinal section in cases where the longitudinal section plane was not perpendicular to the outer enamel surface, the amount being dependent on the sine of the angle of oblique section. From drawings such as Figure 5, *A* and *C*, we can easily determine the true radial enamel thickness at any point on the section planes represented, since we thus incorporate corrections for both longitudinal and transverse curvature. We can be sure that such a measurement will, in fact, be a true radial enamel thickness at that point, even though our original transverse and/or graphical longitudinal section planes may have cut the enamel obliquely. The radial thickness measured from the chosen external enamel

point to the underlying dentin in the transverse sections removes errors resulting from an oblique cut in the tranverse plane. The graphical reconstruction using these measurements allows us to make true radial enamel thickness measurements at any point on the surface. The same reasoning applies with the mesiodistal plane of section.

On the series of reconstructions drawn as in Figure 5, *A* and *C*, for each of the 27 teeth, the radial enamel thickness was measured at 1-inch intervals from the cementoenamel junctions for the four sides, and the results recorded graphically. Since the original enlargement of the sections was 25.4 times, 1 inch on the tracings represented 1 mm. of the original tooth. At the same time, the total incisogingival length of enamel, measured along the outer enamel surfaces, was recorded for each of the four sides. Owing to the loss of tooth structure due to the blade cut, the

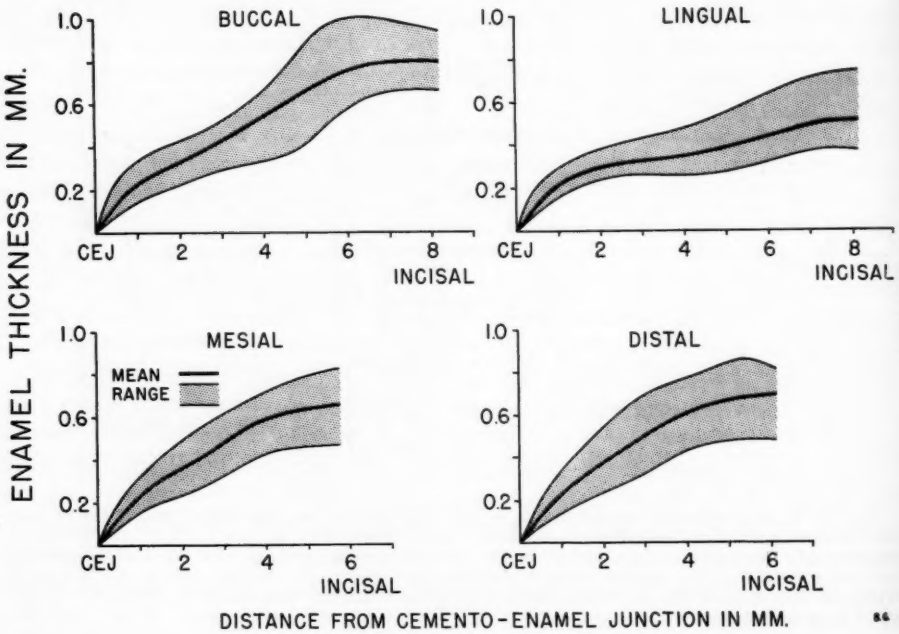


FIG. 6.—Average true enamel thickness distribution curves for the buccal, lingual, mesial, and distal surfaces of 27 lower incisor teeth. Enamel thickness in millimeters is plotted against distance up from cementoenamel junction in millimeters. The mean for 27 teeth is shown by the heavy solid line, and the range by the dotted area in each case.

determination of the exact point where the enamel started or finished was not possible. A mid-point was accordingly used as a base line for incisal and gingival, which gave a maximum base-line error of $+0.15$ mm. at the starting and finishing points of each of the four surfaces, the limits being set by the lower face of the upper section and the upper face of the lower section. This incisogingival measurement is independent of the longitudinal cut adopted, be it straight, curved, or skewed, since this distance is dependent only on the number of sections containing enamel. From the enamel thickness measurements made as described above, graphs were drawn representing the radial enamel thickness versus distance up from the cementoenamel junc-

tion for each tooth. These data were combined to give average curves representing buccal, lingual, mesial, and distal radial enamel thickness versus distance up from the cementoenamel junction to the incisal edge in all 27 teeth and are illustrated in Figure 6. The heavy line represents the average obtained from combining the measurements, while the outer lines indicate the range of values obtained for all 27 teeth. In some cases the extremes were set by one tooth, in some cases by more than one. In order better to visualize the relationship of mean enamel thickness to mean incisogingival length for each surface, the graph illustrated in Figure 7 was constructed, using the same curves. Radial enamel thickness was plotted against distance down from the incisal edge to the cementoenamel junction and enabled us to see immediately the relationships between the lengths and thicknsss of enamel for the 27 teeth studied. (The range of measurements used in establishing the mean incisogingival lengths is presented in Table 1, since to include these ranges in Fig. 7 would be confusing.) Variations in individual tooth size made the presentation of data on the 27 individual teeth difficult. Figure 7 attempts to overcome these variations by representing graphically the average incisogingival lengths for each surface relative to its corresponding average enamel thickness curve.

It is realized that the results represent average values and consequently may not

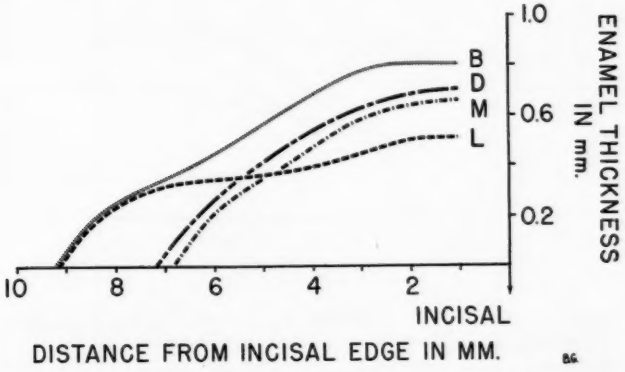


FIG. 7.—Average true enamel thickness distribution curves for the buccal, lingual, mesial, and distal surfaces of the test sample. Enamel thickness in millimeters is plotted against distance down from the incisal edge in millimeters. The range for enamel thickness and incisogingival length are given in Fig. 6 and Table 1, respectively.

TABLE 1

INCISOGINGIVAL LENGTH* OF CROWN SURFACES FOR 27 TEETH (IN MILLIMETERS)

	Mean	Max.	Min.
Buccal	9.15	10.75	7.00
Lingual	9.15	10.40	7.20
Mesial	6.75	7.85	4.80
Distal	7.15	9.20	5.25

* Measured along the outer enamel surface.

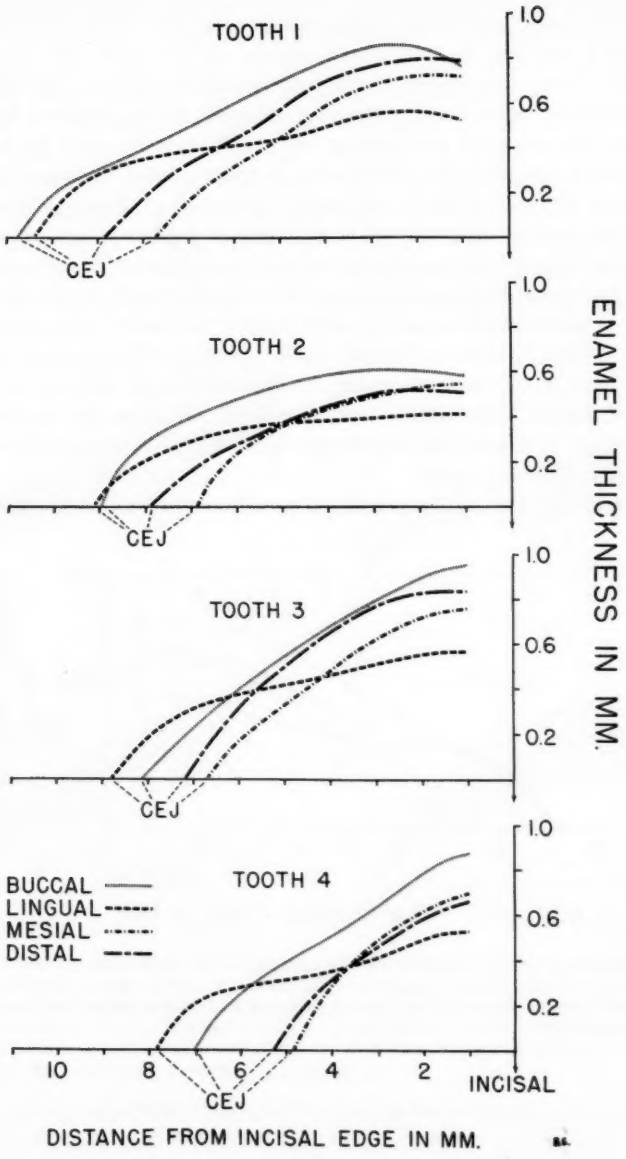


FIG. 8.—Enamel thickness distribution curves for four individual teeth. Individual enamel thickness distribution curves for the four surfaces of four teeth, covering the range in size from large (*tooth 1*) to small (*tooth 4*) illustrate that, while enamel thickness bears no relation to tooth size, the relative enamel thickness distribution for buccal, lingual, mesial, and distal surfaces is similar in each tooth.

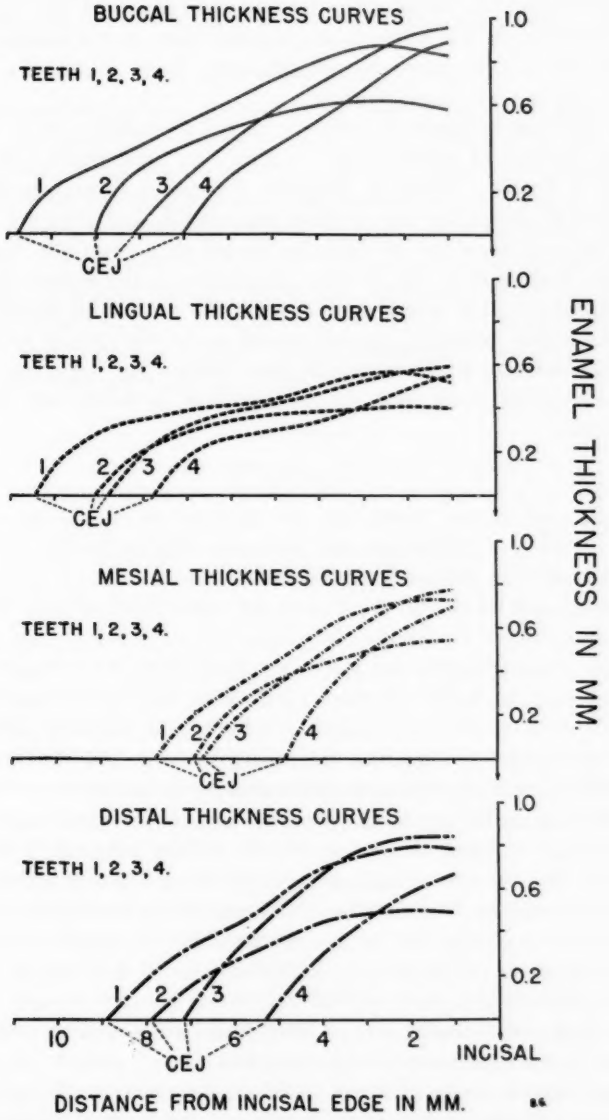


FIG. 9.—Enamel thickness distribution curves for the four surfaces of four individual teeth. The same curves illustrated in Fig. 8 are grouped according to surfaces. Note that the pattern of enamel distribution in a given tooth is preserved for all four surfaces, with minor variations.

be applicable to all teeth. In addition, conclusions drawn from the relationship illustrated in Figure 7 could be criticized on the basis of overlap of the ranges illustrated in Figure 6. However, in the 27 teeth we examined there was a consistent pattern of enamel distribution for the four sides of each tooth. There was no fixed relationship between size of tooth and enamel thickness.

In order to illustrate the pattern, complete enamel distribution curves for four teeth were drawn on two sets of graphs (Figs. 8 and 9), for comparison with the information contained in Figure 7. Figure 8 compares the buccal, lingual, mesial, and distal enamel distribution curves for four teeth of the sample, and Figure 9 compares the same curves, grouped according to surfaces. Crown sizes cover the range from long to short. In the four teeth it can be seen that although the enamel thickness bears no fixed relationship to the actual tooth length, the pattern of enamel distribution and the relative lengths of buccal, lingual, mesial, and distal enamel and points where given curves intersect bear a very nearly constant relationship in a given tooth (Fig. 8) and also from tooth to tooth (Fig. 9). This pattern is maintained throughout the sample of 27 teeth.

RESULTS

In the sample used, it was found that the thickness of enamel did not follow the same pattern on the buccal, lingual, and proximal surfaces. In the average tooth of our sample illustrated in Figure 7, the buccal enamel is seen to be the thickest of all surfaces throughout its length. Starting at the cementoenamel junction, the buccal enamel thickness increases rapidly for about one-sixth of its length and then increases less rapidly and almost linearly for more than half of its total length, maintaining thereafter a constant thickness for the incisal third. The mesial enamel is slightly thinner than the distal for its entire length, and both show a similar pattern of gradually diminishing increase in thickness from gingival to incisal. The lingual enamel increases in thickness quickly, following almost exactly the pattern of the buccal enamel for the gingival sixth of its surface, and then increases in thickness much more slowly than the other three surfaces, and, moreover, for half its length it is the thinnest of all four surfaces. The average incisogingival length of both buccal and lingual enamel is about the same, 9.2 mm., while the mesial and distal incisogingival lengths are 6.8 and 7.2 mm., respectively, for the average tooth of our sample.

Although we have not reproduced the individual enamel distribution curves for all 27 teeth, it was evident that there was no correlation between enamel thickness and crown length. Expressed another way, a long tooth will not necessarily have thick enamel, nor will a short tooth necessarily have thin enamel, and vice versa (see Figs. 8 and 9). There appear to be as many variations between length and thickness of teeth as there are variations between the length and thickness of people.

The sample of 27 teeth was divided into three groups of 9 teeth for convenience in drawing the graphs for each surface. The three sets of average curves of enamel distribution for each surface were remarkably similar, leading to the conclusion that a sample of 9 teeth would give results similar to those of a sample of 27 teeth.

The Student "*t*"-test showed that, at a significance of 10 per cent and using the sample of 27 teeth to estimate the standard deviation, the least number needed to give a mean with a range of ± 0.06 mm. was 9 teeth. These calculations were made

using the enamel thickness measurements from the upper third of the buccal surface (mean 0.75 mm.), where the range of thickness was at a maximum.

DISCUSSION

It was mentioned earlier that teeth showing little or no incisal wear were selected for this study. Although the plane of section used precluded a rigorous investigation of the incisal area, it was possible to arrive at an average crown length and width, using as the criterion the length of the curve representing the buccal enamel surface, from the incisal edge to the cementoenamel junction and the mesiodistal section plane reconstructions. The difference between the average buccal enamel surface length and the average mesial enamel surface length was also recorded. These average values and the range showed close correlation with the values for these surfaces previously published by other authors—Black,[1] Dewey,[3] Diamond,[4] Scott and Symons,[5] and Hopewell-Smith[6]—and are shown in Table 2.*

TABLE 2

CROWN MEASUREMENTS ON LOWER INCISORS

	Av. Buccal Crown Length (Mm.)	Range		Av. Mesio-Distal Crown Width	Range		Av. Buccal Crown Length Minus Av. Mesial Crown Length
		Max.	Min.		Max.	Min.	
Av. of previously published figures	9.18	11.40	6.85	5.83	6.5	4.8	2.5
This study	9.15	10.75	7.00	5.85	6.6	5.1	2.4

It is interesting to note that, while severe incisal wear will affect the measurements of buccal and mesial incisogingival crown lengths, there will be little relative difference between the two. For example, gross wear of 1 mm. would produce less than 3 per cent relative difference in these two lengths, compared with the unworn state. Therefore, the enamel distribution curves resulting from a sample of severely worn teeth would be quite similar to and comparable with the enamel distribution curves obtained from the unworn teeth used in this study.

SUMMARY

A technique has been described for the reproduction of any plane of section in any tooth, or any object, by the use of semiserial sections and a graphical reconstruction technique. This technique was applied to a study of enamel thickness distribution in a pooled sample of 27 lower incisor teeth. A graphical method was used to show that, while correlation between tooth size and enamel thickness was slight, there was a definite pattern of enamel distribution. For the incisal two-thirds of the tooth, the buccal enamel was thickest, followed in turn by the distal, mesial, and lingual enamel.

* Although it is probable that the crown length as expressed by these authors is a straight-line distance from the incisal to the cementoenamel junction as measured by calipers and though the measurements shown in this paper were made along the curve representing the outer surface of the enamel, any differences in the two methods of measurement would be slight, since the greater part of the buccal and mesial surfaces of the lower incisors is a straight line.

The rate of change of enamel thickness from gingival to incisal was quite different for the buccal, proximal, and lingual surfaces.

This technique is equally applicable in investigations of the distribution through teeth of other anatomical characteristics of enamel, such as tufts, lamellae, striae, pigments, optical and radio density, prism orientation, and the spread of dental caries and of physical properties, such as enamel volume, thickness, and surface curvature. The tracings and reconstructions provide an ideal source of data for the study of tooth size and morphology. The technique has already been used in a detailed study of enamel thickness at the base of pits and fissures, since it permitted visualization of an irregular plane of section along the fissure area.[12] A modification of the tracing technique can be used to make accurate large-scale models of teeth and mirror images of such models (i.e., left and right teeth from the one original tooth) for display or teaching purposes.

The technique could be equally well applied to other serial or semiserial sections for reconstruction of various section planes or determination of the three-dimensional configuration of the anatomical features in such structures as cells, blood vessels, and bones.

REFERENCES

1. Black, G. V. *Descriptive Anatomy of the Human Teeth*. 4th ed. Philadelphia: S. S. White Dental Manufacturing Co., 1902.
2. Bodecker, C. F. *Anatomy and Pathology of the Teeth*. Philadelphia: S. S. White Dental Manufacturing Co., 1894.
3. Dewey, M. *Dental Anatomy*. St. Louis: C. V. Mosby Co., 1916.
4. Diamond, M. *Dental Anatomy*. 3d ed. New York: Macmillan Co., 1952.
5. Scott, J. H., and Symons, N. B. *Introduction to Dental Anatomy*. Edinburgh and London: E. and S. Livingstone, Ltd., 1958.
6. Hopewell-Smith, A. *An Introduction to Dental Anatomy and Physiology, Descriptive and Applied*. Philadelphia and New York: Lea & Febiger, 1913.
7. Shaw, J. C. M. *The Teeth and Bony Palate and the Mandible in Bantu Races of South Africa*. London: John Bale Sons & Danielsson, 1931.
8. Tylman, S. D. *Theory and Practice of Crown and Bridge Prosthesis*. 3d ed. St. Louis: C. V. Mosby Co., 1954.
9. Wheeler, R. C. *Textbook of Dental Anatomy and Physiology*. Philadelphia and London: W. B. Saunders Co., 1947.
10. Widdowson, T. W. *Special or Dental Anatomy and Physiology and Dental Histology*, Vols. **1** and **2**. 7th ed. London: Staples Press Ltd., 1946.
11. Gillings, B., and Buonocore, M. An Apparatus for the Preparation of Thin Serial Sections of Undecalcified Tissues, *J. D. Res.*, **38**:1156, 1959.
12. ———. Thickness of Enamel at the Base of Pits and Fissures in Human Molars and Bicuspids, *ibid.*, **40**:119, 1961.

Thickness of Enamel at the Base of Pits and Fissures in Human Molars and Bicuspids

BARRIE GILLINGS and MICHAEL BUONOCORE
Eastman Dental Dispensary, Rochester, New York

The anatomy of pit and fissure areas* in human posterior teeth has long been a subject for dental research. In 1841, W. Robertson, in his book *A Practical Treatise on the Human Teeth*,[2] stressed the possibility of small openings on the occlusal surface leading into large cavities and published a drawing illustrating a fissure extending to the dentin. In 1870 James Truman[3] suggested that fissures predispose to dental caries. A year later J. H. McQuillan[4] made the observation that minute fissures may extend almost to the dentin, and he supported this with an illustration of a ground section showing a fissure leading through a small opening into an oval space in the enamel. In 1899 R. R. Andrews,[5] quoting Tomes, stated: "From the natural depressions which separate the cusps of the molars, minute but deep fissures may be extended through the enamel to within a short distance of the dentin, and they may become larger as they recede from the surface of the tooth." Hopewell Smith[6] in 1926 published a series of articles on enamel with numerous illustrations of deeply invaginated pit and fissure areas. Hyatt and Bodecker[7–19] have both contributed voluminously to the subject and regarded these areas as "faults" in the enamel. Standard texts support this view.[20, 21] More recently, Hodson[22, 23] published several papers on investigations of "T hypoplasia," his term for the deep invaginations he found in posterior teeth, using a technique of slow decalcification, followed by microdissection. He stated that these areas were "manifestations of arrested amelogenesis, often associated with defective matrix formation." Reviews on the pit and fissure area and the associated subject of occlusal caries have been published by Hyatt[12] in 1922 and by Brucker[24] in 1944, the latter containing many references.

Since decay often starts in pit or fissure areas, the presence of deep invaginations of the enamel is thought by many to be an important predisposing factor. Moreover, clinical studies have demonstrated the susceptibility of these areas to caries.[25–28] Methods for caries prevention have been directed at limiting the accumulation of

This study was supported in part by Research Grant D-536 from the N.I.H., U.S. Public Health Service, and in part by the Colgate-Palmolive Company.

Received for publication May 10, 1960; revised by authors July 14, 1960.

* Throughout this report, the terms "pit and fissure area" and "pits and fissures" are used to describe that part of the occlusal surface of a tooth which contains grooves and depressions, whether associated with deep invaginations or not. In some papers and several of the standard texts, sharp distinctions are made between the terms "groove" and "fissure." Dunning and Davenport (see ref. 1) define a groove as "a developmental linear depression or furrow in a tooth surface," and a fissure as "a *deep* [italics ours] ditch or cleft . . . a developmental linear fault . . . commonly the result of the imperfect fusion of the enamel of adjoining dental lobes." The present investigators found it impossible to make this distinction.

foods and the penetration and proliferation of micro-organisms in these areas. Some investigators have applied drugs, such as potassium ferrocyanide, silver nitrate, and sodium fluoride, to the fissure area in the hope that such treatment would retard or prevent dental caries. Others have suggested that materials such as zinc or copper cements, copper or silver amalgams, be applied to the natural fissure for the same purpose. Of all the fissure treatments, the best known is "prophylactic odontotomy," introduced by Hyatt (see above), in which he eradicated caries-susceptible fissures by restoring them with conventional filling materials. This technique was also advocated by Bodecker (see above) but became the subject of vigorous debate for several years, since many failed to comprehend that Hyatt's treatment was not "a mutilating operation on tooth structure,"[29] but rather an immunization of the fissure areas using conservative operative procedures.

While there is good evidence to support the contentions that deep invaginations of the enamel are quite common and that many teeth have areas at the base of pits and fissures where there is little enamel covering the dentin, no detailed studies have been published on the extent to which such areas occur along the length of the principal occlusal fissure. Nor has the actual thickness of the enamel covering the dentin in these areas been reported. The following work was undertaken not only to provide a better understanding of the anatomy of these areas but also as a basis for selection of materials to be used for the prevention of pit and fissure caries and to determine whether a dental probe would permit the clinician to assess the true depth of a fissure.

MATERIALS AND METHODS

For this investigation, 40 caries-free upper and lower first and second molars and 12 caries-free upper first and second bicuspids were used. Owing to the difficulty of including the small auxiliary fissures, only the principal mesiodistal fissure was studied.

It is almost impossible, using existing techniques, to cut an undecalcified tooth section which will contain the whole of this mesiodistal fissure, since this area is seldom a straight line (Fig. 1, *A*). Even if the fissure were straight, any enamel invaginations that might be present in the area would not necessarily be included in such a section, owing to the diverse shapes and directions they may adopt (Fig. 1, *B*). A rigorous examination of the pit and fissure areas by such an approach is thus precluded. However, by making buccolingual serial sections and using a special technique, termed by us "graphical reconstruction," it is possible to represent on co-ordinate paper (using enlarged drawings of these sections) any plane of section, flat, curved or irregular, through the tooth which will include these anatomical features. The technique may be used on any object if (*a*) the object can be serially sectioned at accurately determined, regular intervals and (*b*) the resultant sections can be arranged, after sectioning, in their correct spatial positions. A more detailed consideration of the technique is the subject of another paper.[30]

In this study the teeth were imbedded in rectangular plastic blocks and cut into semiserial sections 130 μ thick at 450-μ intervals on an automatic thin-section machine,[31] the cuts running buccolingually in an axial plane. The molars yielded an average of 24 sections per tooth, while the bicuspids yielded an average of 16 sections per

tooth.* The cut sections were cleansed and mounted in correct order of sectioning, on 3 × 4-inch glass slides, using Canada balsam. Correct order of sectioning and correct orientation of each section in the set were established by the use of reference grooves cut in the rectangular plastic block before sectioning. Three consecutive tracings from the plastic support and tooth section outlines for one of the molars are illustrated in Figure 2, *A*.

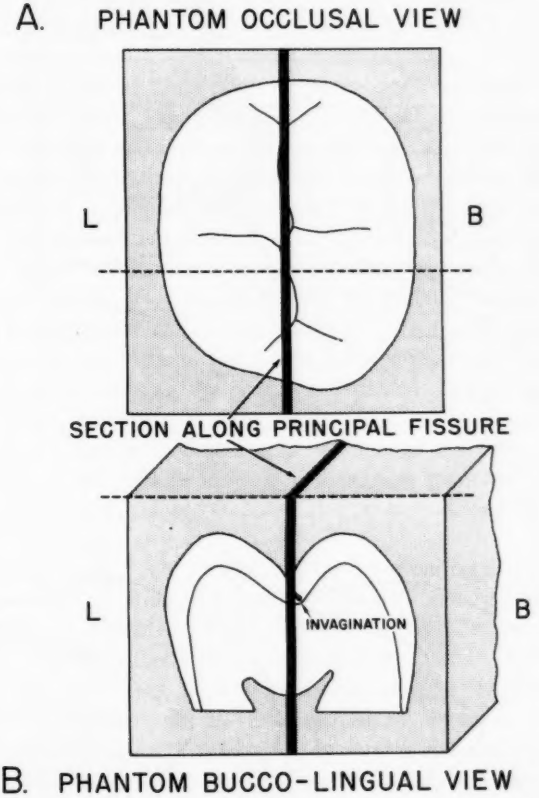


FIG. 1.—Conventional section plane through the fissure area of a molar tooth. (*A*) The conventional mesiodistal section plane (*black area*) cannot follow the often curved or wavy fissure area. (*B*) In areas where the mesiodistal section plane does follow the fissure (e.g., at the level indicated by the horizontal broken line in *A* and *B*), invaginations may not be included in the section.

Using the graphical reconstruction technique referred to above, imaginary planes of section which followed the principal fissure areas in the 52 molars and bicuspids were drawn by reference to these tracings. A numbered series of parallel lines was drawn on co-ordinate paper, spaced 0.45 inch apart, corresponding to each section in each set for

* Mesiodistal crown diameters calculated from these figures (no. of sections × cutting interval [0.45 mm.] = M.D. crown diameter in mm.) agree well with the previously published values of Black, Dewey, Diamond, and Shaw (see refs. 32–35). The mean of their figures for the mesiodistal diameter of pooled molars was 10.7 mm. and of pooled upper bicuspids 7.0 mm. In this study the corresponding mesiodistal diameters of pooled molars was 10.8 mm. and of pooled upper bicuspids 7.2 mm.

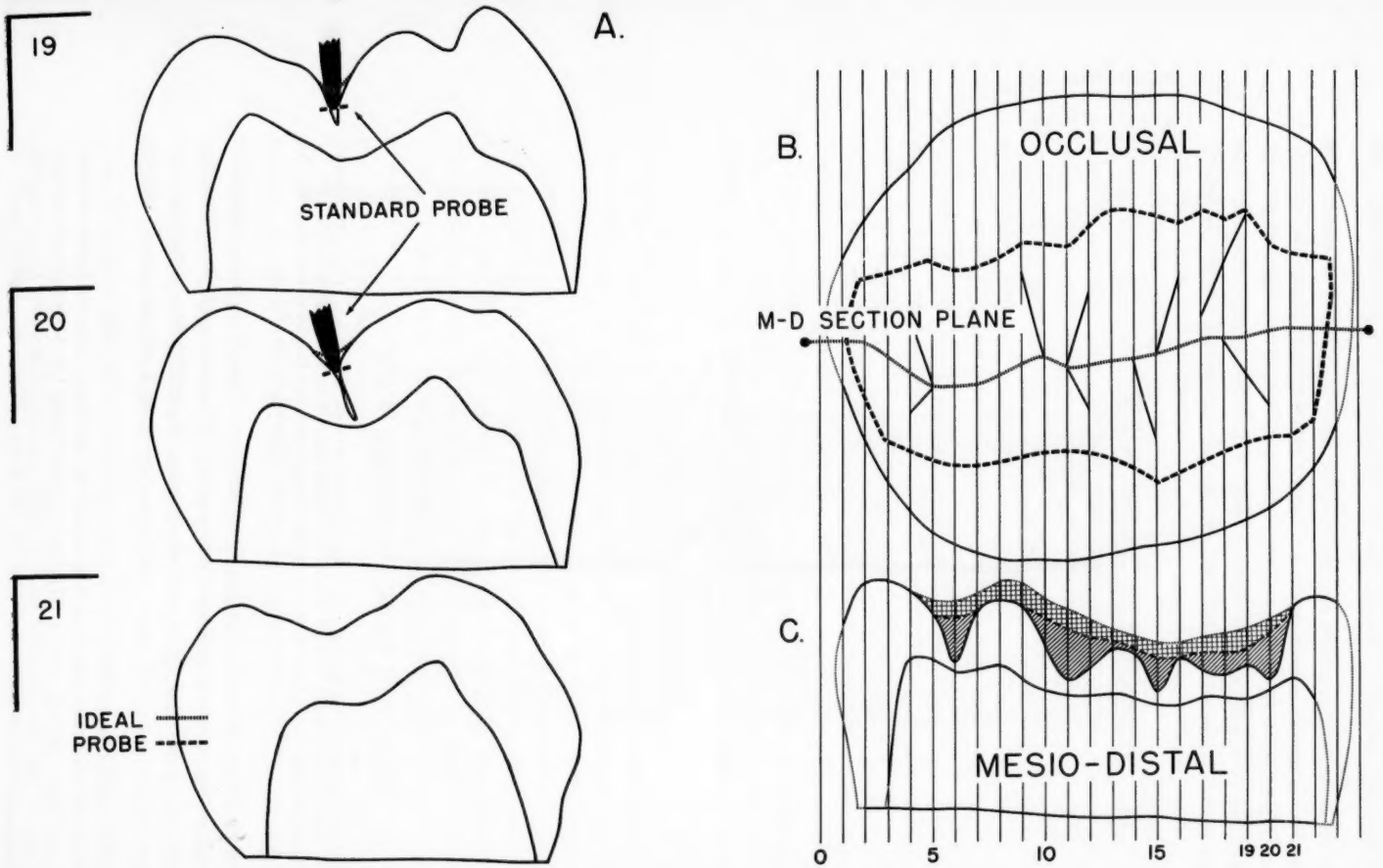


FIG. 2.—Typical tracings and graphical reconstructions. (*A*) Tracings obtained from three consecutive sections of a series of 23 obtained from a lower molar tooth. The corner of each plastic support is identified by the section number. The outline of the probe template is shown in solid black, and its depth of penetration is indicated by the broken line. The outline of the "ideal" enamel surface contour is shown by the dotted line (secs. *19* and *20*). In section *21*, the true, probe, and ideal enamel contours are the same, and the fissure therefore ends at section *20*. (*B*) Occlusal view of a graphical reconstruction of the same tooth, obtained entirely from tracings of the 23 sections. The occlusal surface of the tooth is marked by the broken line, and the course of the mesiodistal fissure is marked by the dotted line. The fine solid outline marks the contour of the crown. The fine solid straight lines mark the positions of the secondary fissures. (*C*) Cross-section view of the mesiodistal fissure shown in *B*, as obtained by graphical reconstruction. True enamel thickness contour at the base of the fissure, together with probe and ideal enamel thickness contours, are shown by the unbroken, broken, and dotted lines, respectively. The difference between probe and true enamel thickness is shown by the diagonally shaded area, and difference between ideal and probe enamel thickness is shown by the crosshatched area.

a given tooth. The points where the imaginary section plane following the fissure would have intersected each individual section were transferred to their appropriate line by using two adjacent sides of each plastic support as base lines for the two co-ordinates, the third co-ordinate being supplied by the section numbers. By this technique it was possible to record the two-dimensional appearance of the fissure area when viewed from the occlusal direction as in Figure 2, *B*, or the buccal direction as in Figure 2, *C*, by simply joining up the points recorded on the parallel lines. The outline illustrated in Figure 2, *C*, is representative of the outline which would have resulted, had it been possible to cut a section to include the whole of the principal fissure area, from mesial to distal, in an axial plane, as illustrated by the dotted line in Figure 2, *B*.

Since we wished to make a comparison between the actual thickness of enamel present in the fissure area and the thickness we would have estimated clinically by using a dental probe (explorer), it was necessary to record the "probe enamel thickness."* This was accomplished as follows. The outline of a sharp explorer was enlarged 25.4 times by means of a projecting microscope† and a template conforming to this shape was constructed. This template was then placed over the tracing of the pit or fissure area of each section in each set, and the distance by which it fell short of the dentinoenamel junction was recorded with calipers (see Fig. 2, *A*). This distance was transferred to the reconstructed plane of section, and the probe enamel thickness along the fissure was traced out by joining the points so obtained with a smooth line, as illustrated in Figure 2, *C*.

In order to have a base-line thickness with which to compare the true enamel thickness and the probe enamel thickness, a similar procedure was used to record the "ideal enamel thickness."‡ Figure 2, *A* and *C*, shows the probe and ideal thicknesses recorded by the procedure outlined above.

The results of this study could be deduced by examination of the 52 reconstructed section planes. However, in order to present the data without reproducing all 52 drawings, the information is presented graphically. Because of differences in size and overall namel thickness between teeth, direct combination of all section planes to produce an "average" section plane was impossible. Deeply invaginated areas occurred in all but one of the teeth examined, but the relative positions of these areas did not follow any definite pattern, even in teeth of apparently similar size and appearance.

The procedure ultimately used to demonstrate the enamel distribution along the fissures of the 52 teeth is most easily explained by analogy. Suppose one were to take several classes of school children of the same grade and have them stand in their classrooms side by side, in order of height. The observer would see that the slopes of lines joining, say, the heads of the students would be comparable from class to class, provided that each class had students with comparable ranges of heights. One could obtain from many classes an average slope of line to show the distribution of heights in

* The term "probe enamel thickness" is the term used in this paper to describe the enamel thickness that would have been indicated by a sharp probe, assuming that the depth to which it penetrated would indicate the bottom of the pits and fissures.

† Bausch and Lomb Tri Simplex Micro Projector.

‡ The term "ideal enamel thickness" as used in this paper describes the thickness of enamel that would have been present in the fissure area, had the buccal incline of the lingual cusp merged smoothly with the lingual incline of the buccal cusp or, expressed another way, if the enamel surface outline followed the outline of the underlying dentinoenamel junction (see Fig. 2, *A*).

this grade. In essence, this is how the enamel thicknesses for an "average tooth" were recorded.

The true enamel thickness at each section level of each graphically constructed section plane was measured, and a number assigned to it, from the thinnest to the thickest. Using co-ordinate paper on which the ordinate paper on which the ordinate represented enamel thickness and the abscissa the numbers assigned to each section, the true enamel thickness measurements were recorded. The thickness distribution curve (Fig. 3) resulting when these points were joined with a smooth line is shown for the tooth previously illustrated in Figure 2. (As the sections were cut at 450-μ intervals, the divisions on the abscissa can also be used to indicate total fissure length for each tooth.) For each tooth similar curves were constructed, showing the probe enamel thickness and the ideal enamel thickness, by the same procedure. Average curves for these three thicknesses were then constructed. For convenience in handling the data,

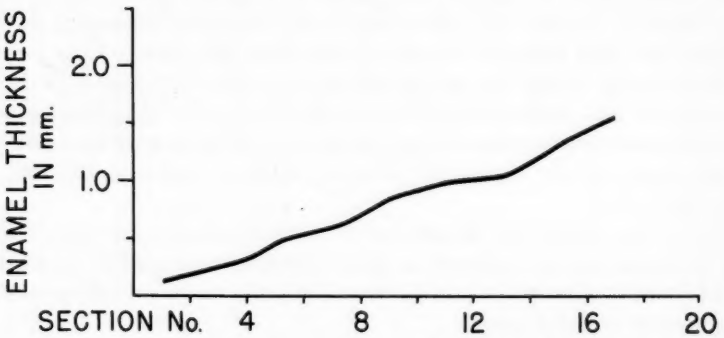


FIG. 3.—True enamel thickness distribution curve for a molar tooth. Thickness distribution curve for the tooth illustrated in Fig. 2, where numbers are assigned to sections arranged in order of increasing enamel thickness and plotted against the enamel thickness in millimeters at each section level. Each section number represents 450 μ of the fissure area.

the molar sample was divided into four groups of 10 teeth, and average curves were drawn. (Each point on the abscissa corresponding to a section number therefore represented an average of 10 measurements.) Mean curves representing true, probe, and ideal enamel thickness for the sample of 40 teeth were obtained by combination of the data recorded in the relevant sets of four average enamel thickness distribution curves. The bicuspid sample was similarly handled, but as one group of 12 teeth.

RESULTS AND DISCUSSION

For purposes of comparison, the average curves for true enamel thickness, probe enamel thickness, and ideal enamel thickness for the 40 molar teeth are shown in Figure 4, and for the 12 bicuspid teeth in Figure 5. The range of the measurements for the true enamel thickness is shown in Figures 6 and 7.

It was evident from examination of the 1,312 sections prepared from these 52 teeth that almost every tooth fissure exhibited deeply invaginated areas. In many cases these areas went almost to the dentinoenamel junction (see Figs. 8–13). However, in none of these cases did the dentin suface appear to be exposed. From examination of the reconstructed fissure section planes, together with their probe and ideal enamel

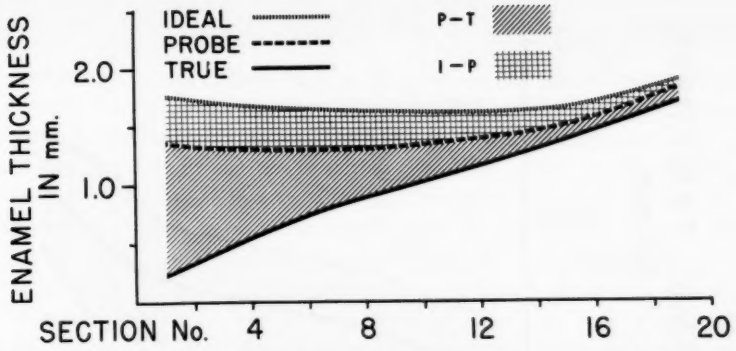


FIG. 4.—Average curves for enamel thickness of molars. The solid line represents the average true enamel thickness distribution curve obtained from averaging the enamel thickness curves of the 40 molar teeth. The broken and dotted lines represent the average probe and ideal enamel thickness distribution curves, respectively, obtained in a similar fashion. The difference between the average probe and true enamel thickness is shown by the diagonally shaded area, and the difference between the ideal and probe enamel thickness is shown by the crosshatched area.

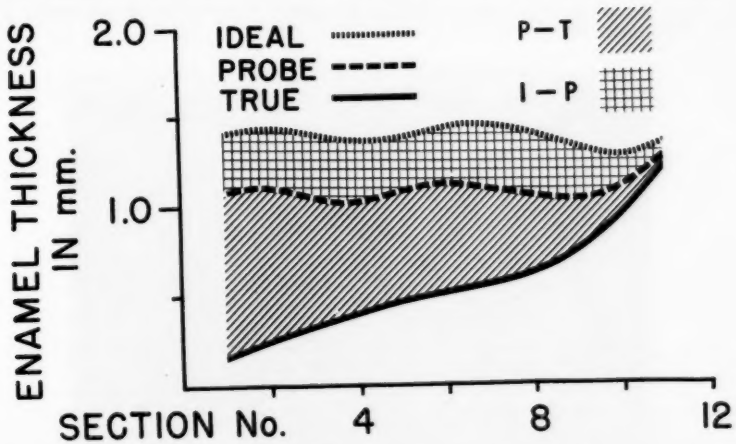


FIG. 5.—Average curves for enamel thickness of upper bicuspids. The solid line represents the average true enamel thickness distribution curve obtained from averaging true enamel thickness curves for each of 12 upper bicuspid teeth. The broken and dotted lines represent the average probe and ideal enamel thickness distribution curves, respectively, obtained in a similar fashion. The difference between the average probe and true enamel thickness is shown by the diagonally shaded area, and the difference between the ideal and probe enamel thickness is shown by the crosshatched area.

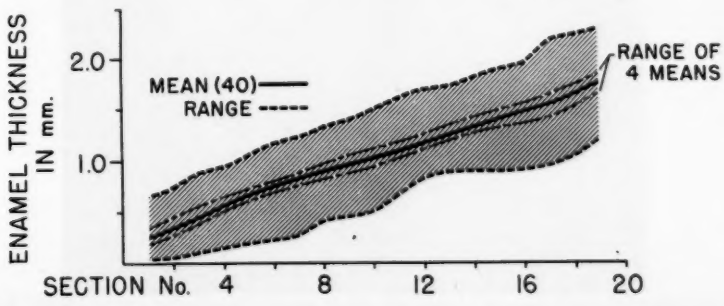


FIG. 6.—Range of measurements for true enamel thickness of molars. The average true enamel thickness distribution curve for the sample of 40 molars is shown by the solid line. The total range of measurements encountered in the sample of 40 molars is shown by the diagonally shaded area, bounded by the heavy broken lines. The range for four means obtained by dividing the group of 40 teeth into four groups of 10 teeth and averaging separately is shown by the fine broken lines (see text).

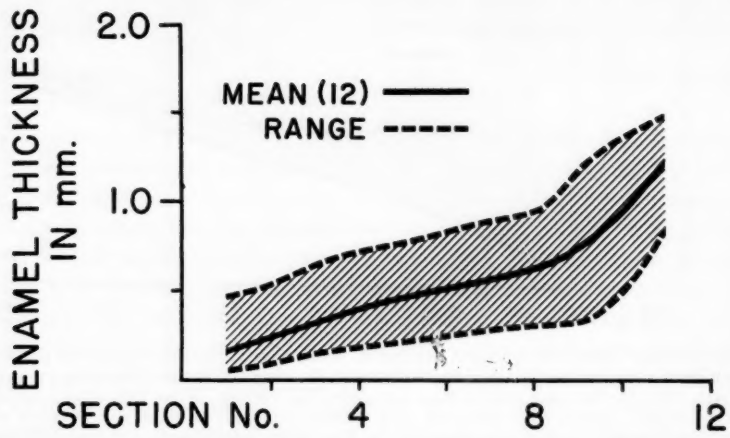


FIG. 7.—Range of measurements for true enamel thickness of upper bicuspids. The average true enamel thickness distribution curve for the sample of 12 upper bicuspids is shown by the solid line. The total range of measurements encountered in the sample is shown by the diagonally shaded area bounded by the broken lines.

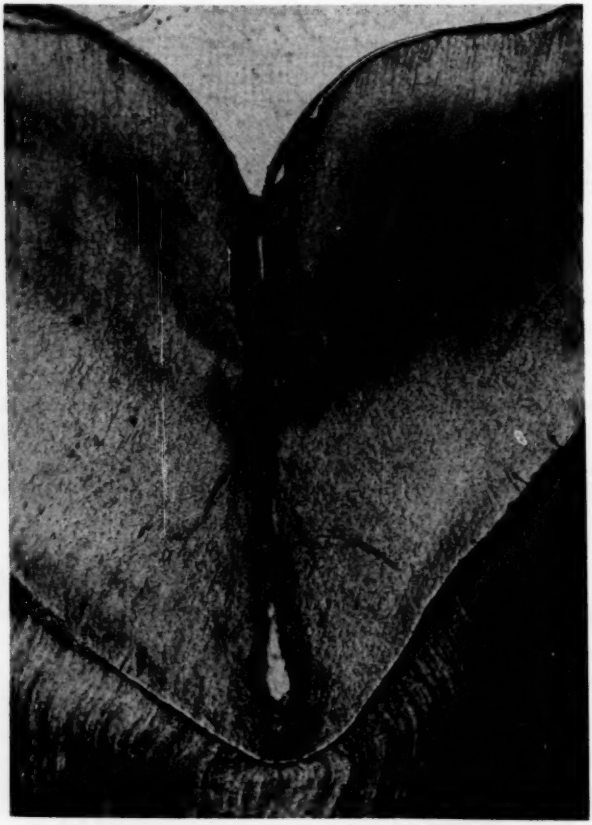


FIG. 8.—A typical deeply invaginated molar fissure

thickness recordings, it was evident that in all cases but one any attempt to gauge the enamel thickness present at the base of a pit or fissure by the use of a probe may give the clinician a misleading impression. The location of the invaginated areas did not appear to follow any definite pattern in any of the molars, even when comparing teeth from the same position in the mouth, although they did occur to about the same extent in both upper molars, lower molars, and upper bicuspids. The deepest invaginations tended to occur in the region of the mesial, central, and distal pits.

From the average thickness curves obtained as described previously, the following information was obtained: For the average molar in this study, true enamel thickness of less than 1.0 mm. was present over half the fissure area, while probe enamel thickness was more than 1.3 mm. over the same area. For one-third of the fissure length, the true enamel thickness was under 0.7 mm. and less than half the probe enamel thickness. In general, the ideal enamel thickness was about 0.3 mm. greater than the probe enamel thickness (see Fig. 4).

It is interesting to note that the four average true enamel thickness curves for the molars so closely resembled one another that they almost fell on the same line when

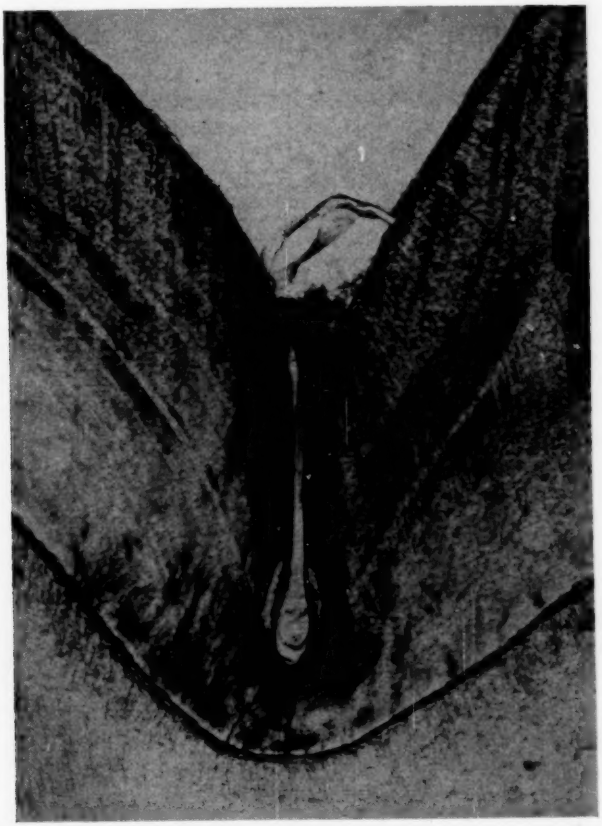


FIG. 9.—A typical deeply invaginated fissure in an unerupted bicuspid. Note the presence of organic matter.

drawn on the one graph, as can be seen from the range of the four means illustrated in Figure 6. A similar situation applied to the curves in the other two sets of probe and ideal thickness curves, but not to the same extent. This evidence suggests that a sample of 10 molar teeth would give results similar to a group of 40 molar teeth. The student "*t*"-test was used to determine the minimum number of teeth needed to give a mean true enamel thickness with a range of ± 0.15 mm. at a significance of 10 per cent, using the sample of 40 molar teeth to establish the standard deviation, for section level 16 (see Fig. 6), where the range is greatest. The minimum number of teeth needed was found to be 10, and the range of means obtained from four groups of 10 is consistent with this finding (see Fig. 6). In a fissure area, the probe enamel thickness will always be more than the true enamel thickness, and the ideal enamel thickness will always be more than the probe enamel thickness. Any observed differences in these thicknesses must therefore be real and do not require statistical evaluation. There was little or no difference in the extent to which deeply invaginated areas occurred in the various types of molar teeth examined. We used a small sample of only 12 teeth for the bicuspid study, since we believed that this small sample would probably pro-

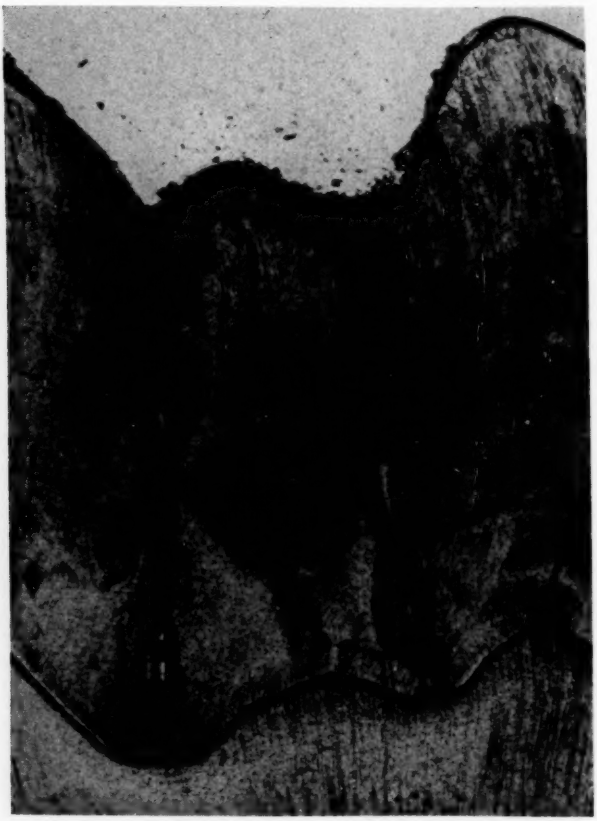


FIG. 10.—Deep invaginations in a molar. The principal fissure showed a Y-shaped division just before this section level.

vide a good indication of the relative frequency of these invaginated areas in upper bicuspid teeth in general. We are currently sectioning more bicuspids to test this belief.

For the average bicuspid in this study, the true enamel thickness was less than 0.5 mm. for over half the fissure length, while the probe enamel thickness was more than 1.0 mm. over the same area. For two-thirds of the fissure length, true enamel thickness was less than half the probe enamel thickness. In general, the ideal enamel thickness was about 0.3 mm. greater than the probe enamel thickness.

While the lines on the graphical reconstructions representing the enamel contour at the base of the fissure for each single tooth bore no resemblance to the contour of the dentinoenamel junction, it was evident that in nearly all the tracings the contour of the probe enamel thickness and ideal enamel thickness closely resembled each other and the contour of the underlying dentinoenamel junction (see Fig. 2, *C*). This means, in effect, that an impression in a viscous material, such as dental compound,* of the occlusal surface of a molar tooth would give a general indication of the contour of the dentinoenamel junction underlying the fissure.

* Because of its poor flow, such a material would reproduce a groove, but not a fissure, using the classical definition of these terms.

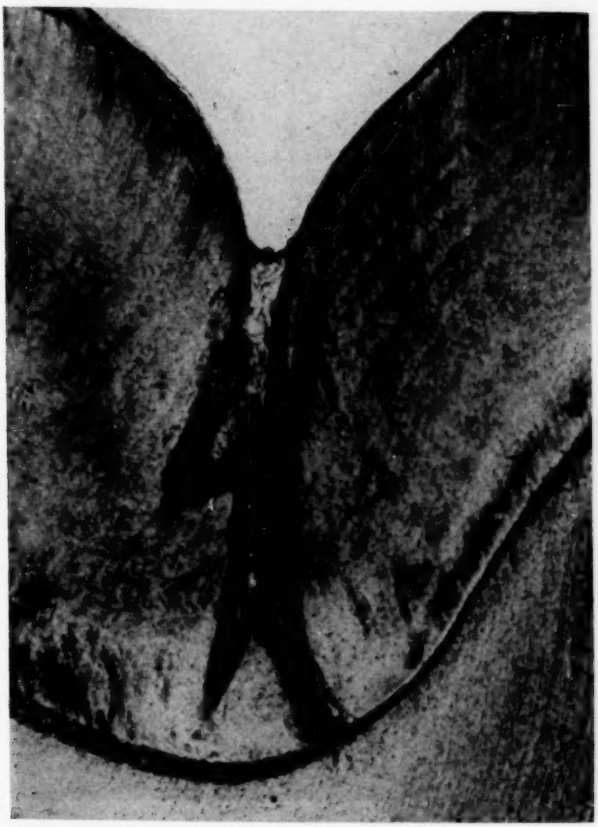


FIG. 11.—Multiple invaginations occasionally encountered in molar teeth

The most significant finding in this study was the frequency and extent of deep, inaccessible areas in the fissures of posterior teeth. Photomicrographs of some typical and unusual deep invaginations are illustrated in Figures 8–13. The graphical reconstructions only indicate the appearance of these areas when viewed from the buccal (or lingual) aspect and give no indication of the width of these areas in a buccolingual direction. From an examination of the sections it was evident that in many cases these invaginations had marked constrictions close to the occlusal surface and opened into quite extensive cavities deep in the enamel. These areas would be ideal locations for the proliferation of micro-organisms and would resist efforts to clean them.

Included in both samples were some unerupted teeth. The fissures from such teeth invariably contained organic matter, presumably the remnants of the enamel organ. The fissure from one unerupted bicuspid contained what appeared to be bone (see Fig. 13). Most of the erupted teeth contained pigmented debris in these areas, probably consisting of food products, microorganisms, and remnants of the enamel organ.

Since the molar teeth used in this study were caries-free and some were from elderly subjects, the presence of deeply invaginated enamel areas cannot be the sole reason

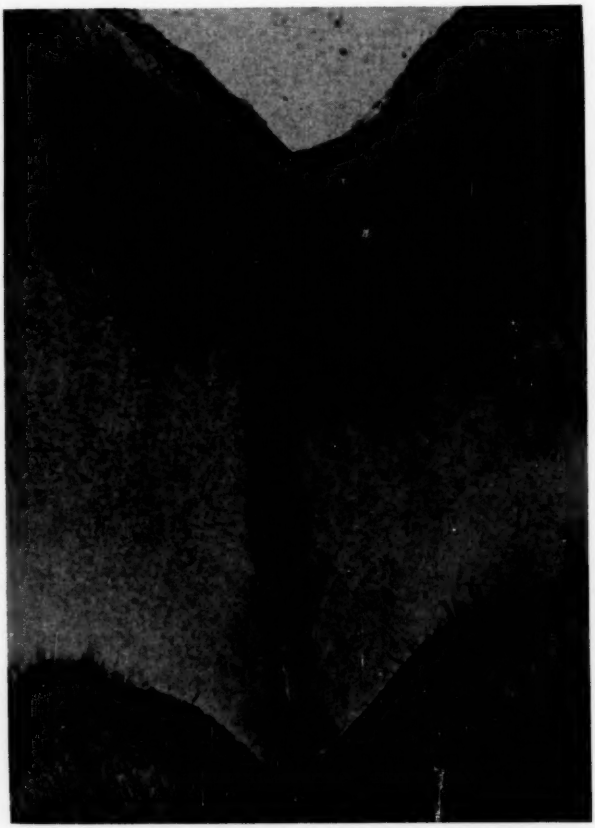


FIG. 12.—Very occasionally a transverse section will not include all the invagination, as in this case. The plane of section here did not correspond to the plane of the invagination for its entire length.

for the early decay often observed clinically in molar teeth. Nevertheless, the present investigators feel that if a sample of newly erupted caries-free posterior teeth had been available, they would perhaps have shown these invaginations to an even greater extent, since the presence of such areas is considered strongly predisposing to caries. The molar sample used by the authors is probably more representative of caries-resistant teeth than of molar teeth in general. All the bicuspids used were unerupted or newly erupted, having been extracted for orthodontic reasons, and are more likely to be representative of upper bicuspids in general.

SUMMARY

The pattern of distribution of deeply invaginated pit and fissure areas and the enamel thickness at the base of these areas in a pooled sample of 40 upper and lower first and second molars and 12 upper first and second bicuspids was investigated, using 1,312 undecalcified semiserial sections and a graphical reconstruction technique, as follows: (*a*) imaginary planes of section which followed the fissure area of each tooth were drawn on co-ordinate paper, from measurements obtained from enlarged tracings

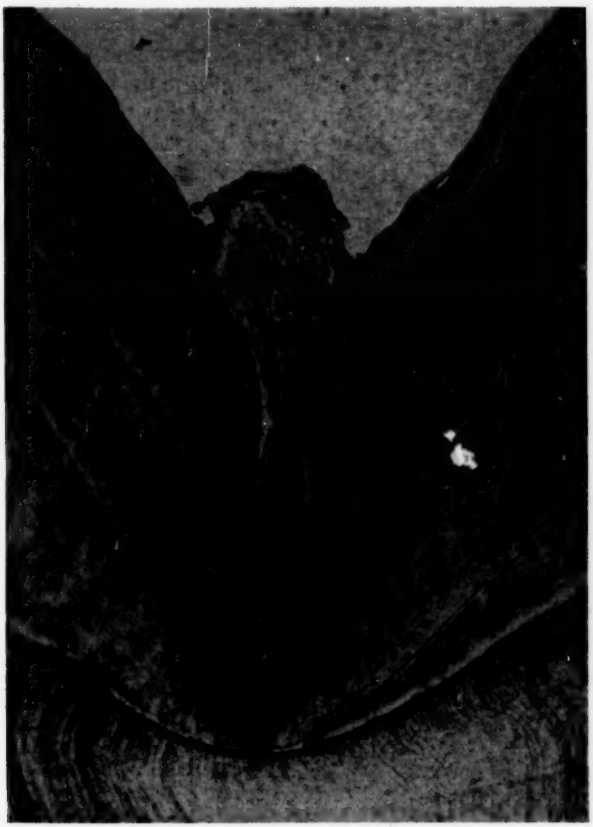


FIG. 13.—A section from one of the two unerupted teeth which contained what appeared to be bone in the fissure.

of each of the 1,312 sections; (*b*) from these planes and their associated sections, three graphs were drawn for each of the 52 teeth, representing true enamel thickness, probe enamel thickness, and ideal enamel thickness, along the length of each fissure; (*c*) these individual graphs were combined to construct average curves, which showed the distribution of enamel thickness along the length of a fissure.

In all but one of the 52 teeth examined, the presence of deeply invaginated fissure areas was noted. Although there was no fixed relationship between tooth morphology and distribution of these areas, they were present to about the same extent in the molar and upper bicuspid teeth sectioned. The use of a dental probe to gauge the thickness of enamel in such areas is bound to give the clinician a misleading impression, since the thickness estimate could be optimistic by a factor of six times in the molars and five times in the bicuspids.

The contour of the occlusal opening of the fissure corresponded to the contour of the underlying dentinoenamel junction but not to the contour of the enamel surface along the fissure base.

This study shows that areas of deep invagination in the enamel are much more prevalent than was hitherto suspected. If a tooth has an occlusal surface containing pits and/or fissures which will admit even the point of a probe, there is every likelihood that such a tooth will contain some areas of deeply invaginated enamel. Our investigations of these areas lead us to the conclusion that the presence of these areas in teeth is normal and tends to discount the contention that they are structural malformations or the result of some hypoplastic condition or developmental anomaly.

The fact that teeth can be free of caries despite the presence of these areas is worthy of further investigation of possible factors responsible for this apparent immunity. Furthermore, the widespread existence of these areas, coupled with the high and early incidence of decay in the pit and fissure areas of posterior teeth, suggests the need for research to develop materials which could be applied dirctly to the natural fissure, to seal over these ubiquitous food traps, somewhat along the lines of Hyatt's original suggestion but without the operative work involved.

REFERENCES

1. Dunning, W. B., and Davenport, S. E. *A Dictionary of Dental Science and Dental Art.* Philadelphia: P. Blakiston Son & Co., 1936.
2. Robertson, W. *A Practical Treatise on the Human Teeth.* 1st American ed. Philadelphia: Lea & Blanchard, 1841.
3. Truman, J. Fissures, *D. Cosmos,* **12**:1, 1870.
4. McQuillan, J. H. Microscopical Fissures in the Masticating Surface of Molars and Bicuspids, *D. Cosmos,* **13**:300, 1871.
5. Andrews, R. R. The Pits and Fissures of the Enamel, *D. Cosmos,* **31**:719, 1899 (abstr.).
6. Hopewell-Smith, A. Concerning Human Enamel: Facts, Explanations and Applications, *D. Cosmos,* **68**:639, 1926; **69**:360, 468, 1927.
7. Hyatt, T. P. Prophylactic Odontotomy, *D. Cosmos,* **65**:234, 1923.
8. ———. Some Further Considerations of the Possibilities of Preventive Measures for the Prevention of the Entrance of Caries into First and Second Permanent Molars through Occlusal Pits and Fissures, *D. Cosmos,* **65**:623, 1923.
9. ———. Occlusal Fissures, How Shall They Be Treated, *D. Items of Interest,* **46**:493, 1924.
10. ———. Prophylactic Odontotomy, *J. D. Res.,* **6**:389, 1925.
11. ———. The Conservation of Non-Caries First Permanent Molars, *J.A.D.A.,* **13**:655, 1926.
12. ———. A Review of the Dental Literature on Pits and Fissures, *J. D. Res.,* **10**:659, 1930.
13. ———. A New Aspect of Prophylactic Odontotomy, *J.A.D.A.,* **18**:1356, 1931.

14. ———. Prophylactic Odontotomy, the Ideal Procedure in Dentistry for Children, *D. Cosmos,* **78**:353, 1936.
15. Bodecker, C. F. Microscopic Study of Enamel Fissures, with Reference to Their Operative Treatment, *D. Cosmos,* **66**:1054, 1924.
16. ———. The Toothbrush in Relation to Enamel Caries, *D. Items of Interest,* **48**:161, 1926.
17. ———. Bacteria in Relation to Enamel Caries, *ibid.,* p. 797.
18. ———. The Eradication of Enamel Fissures, *ibid.,* **51**:859, 1929.
19. ———. The Cause of the Rapid Decay of Occlusal Enamel Fissures, *ibid.,* p. 21.
20. ———. *Fundamentals of Dental Histology and Embryology, Including Clinical Applications.* New York: Columbia University Press, 1944.
21. Kronfeld, R. *Histopathology of the Teeth and Their Surrounding Tissues.* Philadelphia: Lea & Febiger, 1949.
22. Hodson, J. J. Tubular Invaginations of the Enamel Capsule, *Brit. D. J.,* **87**:167, 1949.
23. ———. A Study of Some of the Developmental, Structural and Pathological Aspects of Tubular Hypoplasia in Human Enamel, *ibid.,* **89**:6, 34, 1950.
24. Brucker, M. Studies on the Incidence and Cause of Dental Defects in Children. VI. Pits and Fissures, *J. D. Res.,* **23**:89, 1944; VII. Fissures and Caries, *ibid.,* p. 101.
25. Brekhus, P. J. A Report on Dental Caries in 10,445 University Students, *J. D. Res.,* **11**:487, 1931 (abstr.).
26. McCall, J. O. Lessons To Be Learned in a Children's Dental Clinic, *J.A.D.A.,* **21**:1869, 1934.
27. Prime, J. M. Inconsistencies in Operative Dentistry, *J.A.D.A.,* **24**:82, 1937.
28. ———. Controlling Dental Caries, *ibid.,* p. 1950, 1937.
29. Kirk, E. C. Prophylactic Odontotomy, *D. Cosmos,* **65**:319, 1923 (editorial).
30. Gillings, B., and Buonocore, M. An Investigation of Enamel Thickness in Human Lower Incisor Teeth, *J. D. Res.,* **40**:105, 1961.
31. ———. An Apparatus for the Preparation of Thin Serial Sections of Undecalcified Tissues, *ibid.,* **38**:1156, 1959.
32. Black, G. V. *Descriptive Anatomy of the Human Teeth.* 4th ed. Philadelphia: S. S. White Manufacturing Co., 1902.
33. Dewey, M. *Dental Anatomy.* St. Louis: C. V. Mosby, 1916.
34. Diamond, M. *Dental Anatomy,* 3d ed. New York: Macmillan Co., 1952.
35. Shaw, J. C. M. *The Teeth and Bony Palate and the Mandible in Bantu Races in South Africa.* London: John Bale Sons & Danielsson, 1931.

Bacterial Invasion of Non-vital Dentin

I. M. CHIRNSIDE

University of Otago Dental School, Dunedin, New Zealand

Bacteria have been reported in the tubules of non-vital dentin in the absence of caries. Rushton[1] reported their presence in some tubules opening off deep cracks in tracts of dead dentin exposed to the mouth by wear, and Hodson[2] observed bacteria in dentin of a prematurely exposed, hypocalcified permanent incisor of a child with gross oral sepsis who died at the age of one year and eleven months. The present author[3] has demonstrated bacteria deep in dentinal tubules around infected root canals.

While these examples show that bacteria may invade odontoblastic processes of dead dentin in special environments, they leave unanswered the question whether processes of dead dentin exposed to the mouth would be similarly invaded. This was investigated by mounting and wearing suitably prepared extracted teeth in a specially designed partial denture.

MATERIALS AND METHODS

Immediately following extraction of a sound premolar, dentin on the occlusal surface was exposed by grinding. The dentin was then roughened slightly with a bur to produce a surface which would later favor formation of a plaque. At the amelocemental junction a depression was ground for the full circumference of the root. This depression was scrubbed with tincture of iodine several times prior to dividing the tooth at this point with a sterile diamond disk. The root was discarded and pulp tissue removed from the coronal portion against the possibility that bacteria forced into the pulp chamber during extraction might thrive and invade the odontoblastic processes from the pulp.

The entry to the pulp chamber was then sealed with a little wax prior to imbedding the crown in zinc phosphate cement in a trough provided in the molar region of a partial lower denture. Imbedding was done in a manner which left only the uncovered dentin of the occlusal surface exposed to the mouth. The denture was worn continuously throughout the experimental period, and during that time the exposed dentinal surface was rinsed but not otherwise cleaned.

At the end of the period the crown was removed from the denture, the pulp chamber unsealed, and the crown immersed in formol-saline. Paraffin sections were prepared at 6 μ in the long axis of the crown and in the buccolingual plane. Of those sections which disclosed a portion of the pulp chamber, every third one was stained by Gram's method and examined for the presence of bacteria in the dentinal tubules. Measurement of sections showed the thickness of dentin overlying the pulp chamber to vary between 1 and 2 mm. At different times, five teeth were so treated; three were exposed for 4 weeks, one for 3 weeks, and one for 5 weeks. As a control, five teeth were treated similarly, but, unlike the experimental series, occlusal enamel was not removed.

Received for publication May 24, 1960; revised by author August 22, 1960.

RESULTS

Table 1 shows the incidence of bacteria in dentinal tubules of the experimental and control groups to be in the ratio of 5:1. This result is significant at the 0.02 level (Fisher-exact probability test).

Microscopic examination of the experimental series disclosed in every case a heavily infected plaque attached to the exposed dentinal surface (Fig. 1). From this, bacteria had invaded some odontoblastic processes in the manner shown in Figures 2 and 3. They could be traced, though not with complete continuity, from the exposed occlusal surface to the pulpal end of the dentinal tubules (Figs. 4 and 5). The invasion had a random distribution but was confined to tubules subjacent to the exposed dentinal surface. In the one control where bacteria were observed in tubules, they stemmed from a colony established on the pulpal surface of dentin just inside the entry to the pulp chamber. They were not related to the exposed occlusal surface. Their presence was attributed to contamination either during extraction or during the experimental procedure.

The results justify the conclusion that if odontoblastic processes succumb following

TABLE 1

INCIDENCE OF BACTERIA IN DENTINAL TUBULES

Group	No.	Positive	Negative
Experimental	5	5	.....
Control	5	1	4

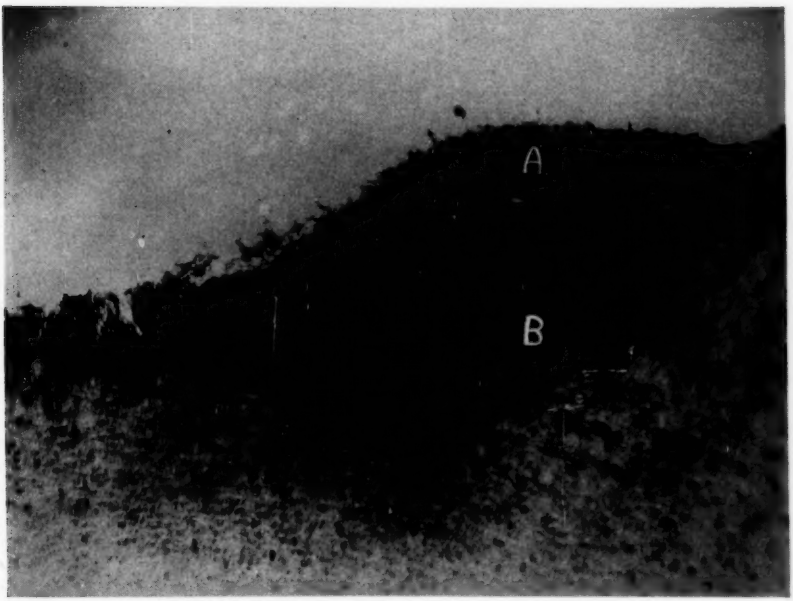


FIG. 1.—Non-vital dentin exposed to mouth. *A* = plaque; *B* = dentin. (Mag. ×60.)

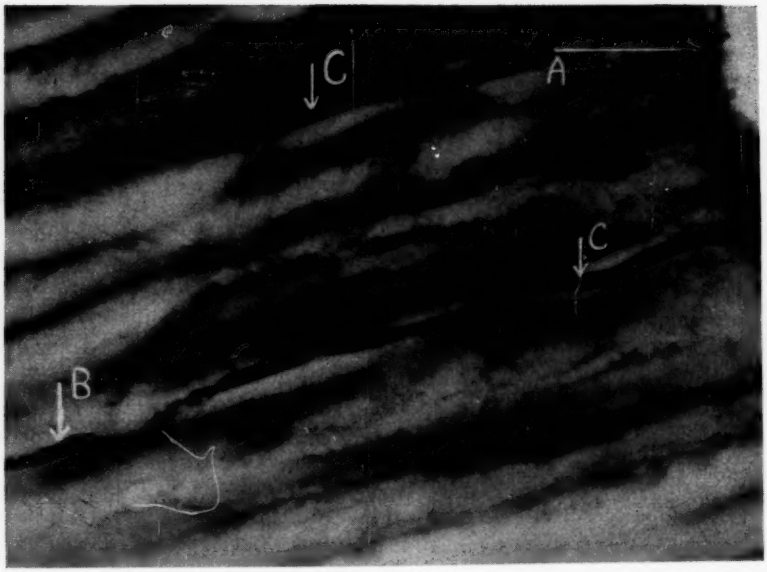


FIG. 2.—Non-vital dentin exposed to mouth. *A* = bacterial plaque; *B* = bacteria invading odontoblastic process; *C* = bacteria out of focus. (Mag. ×1,500.)

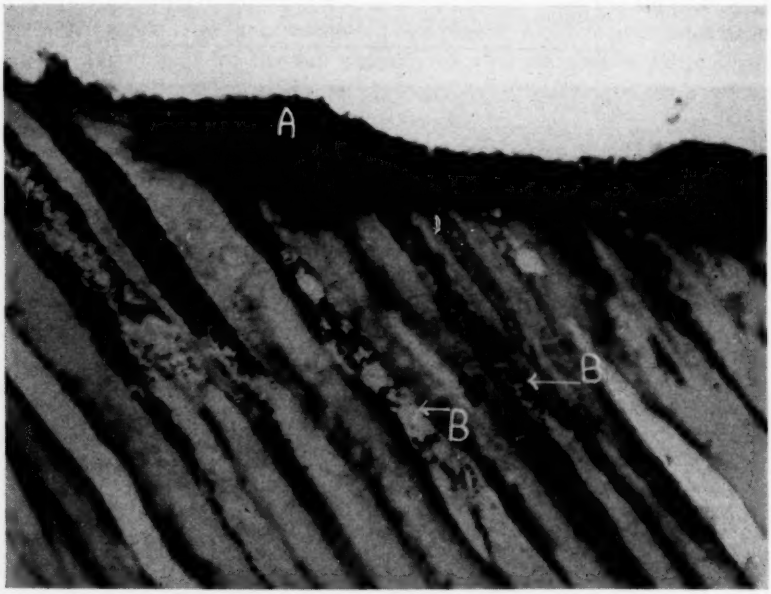


FIG. 3.—Non-vital dentin exposed to mouth. *A* = bacterial plaque; *B* = bacteria invading odontoblastic processes. (Mag. ×1,500.)

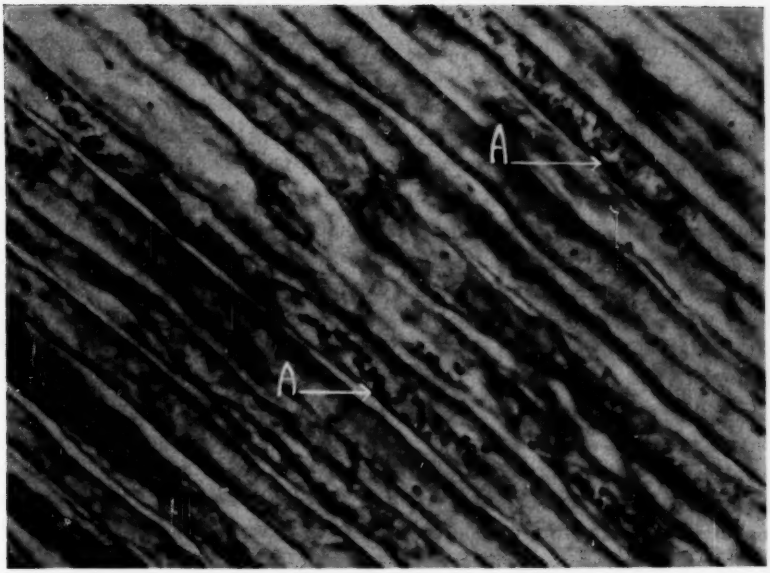


FIG. 4.—Non-vital dentin exposed to mouth. *A* = bacteria deep in dentin. (Mag. ×1,500.)

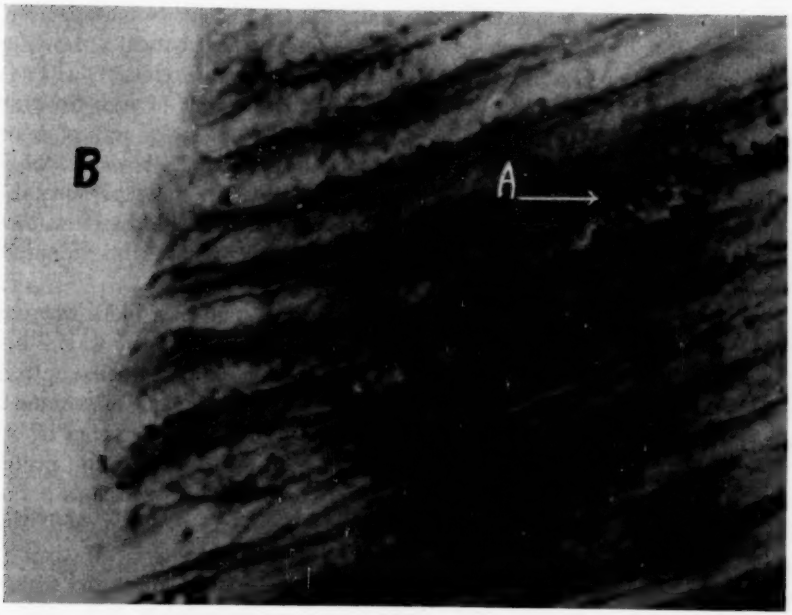


FIG. 5.—Non-vital dentin exposed to mouth. *A* = bacteria; *B* = empty pulp chamber. (Mag. ×1,500.)

death of the pulp or peripheral injury and are exposed to the mouth in circumstances which allow formation of a plaque, they are liable to bacterial invasion.

DISCUSSION

This conclusion has a bearing on several issues. Whether bacteria precede or follow decalcification in dental caries is a case in point. Kronfeld,[4] for example, noted that under a carious lesion there were frequently a few tubules containing bacteria well in advance of decalcification of the matrix. Zander[5] confirmed this and suggested that decalcification precedes bacterial invasion in acute caries and that in chronic caries the relationship is reversed. MacGregor, Marsland, and Batty,[6] too, found bacteria in advance of decalcification, though only in a minority of cases.

On the other hand, Dorfman, Stephan, and Muntz[7] and Sognnaes and Wislocki[8] concluded that decalcification preceded bacterial invasion in caries. Dorfman *et al.*[7] attributed the conflict in this issue to the impossibility of discriminating in decalcified sections between decalcification due to caries and that due to histologic technique.

It could well be that, for mineralized tooth substance, decalcification is an essential preliminary to bacterial progress. Dentin, however, consists of a mineralized matrix permeated by unmineralized odontoblastic processes. Fish[9] has shown that under caries some processes are dead, and the present work shows that necrotic odontoblastic processes may be invaded quite independently of decalcification of surrounding matrix. It is suggested, therefore, that the presence of bacteria in some tubules in advance of decalcification is not surprising and is due to a saprophytic invasion of odontoblastic processes which have succumbed, in part or whole, to the carious attack. What influence this has on the progress of the lesion is problematical.

The vulnerability of necrotic odontoblastic processes is also relevant to the fate of teeth whose pulps die following a blow. Even when the crowns of such teeth are not injured, the pulps of many become infected,[10, 11] it is thought, by way of either the blood stream or the lymphatics of the periodontal membrane. If, however, the dentin of the crown is exposed, the rapid invasion of processes in the experimental series leaves little doubt that, in the absence of previously laid calcific barriers, the dead pulp of a traumatized tooth may be invaded by way of exposed necrotic odontoblastic processes.

Nor should the possibility be discounted that a dead pulp in a tooth whose crown remains intact may be similarly invaded, though in the first stages, by way of infected lamellae. If Manley and Hardwick's[12] doubt of the extension of lamellae into dentin is confirmed, the existence of enamel tufts, spindles, and basement membrane might still provide an organic bridge from lamellae to the terminal ramifications of odontoblastic processes, which in some instances might be large enough to admit proteolytic organisms.

Fish[13] found that under caries, with a few exceptions, tubules containing severely damaged odontoblastic processes were sealed with calcific material at their pulpal extremity. The pulp opposite the few exceptions, however, was infiltrated with round cells, and one such tooth had a long history of hypersensitivity. Both features he attributed to infiltration of toxic fluids from the mouth. Both phenomena, it is suggested, might have been due rather to bacteria which in those particular tubules had

reached and were then being nourished by pulp, bearing the same relationship to it as bacteria in an infected root canal bear to periodontal membrane.

Similarly, it is probable that some tubules containing processes of odontoblasts injured by cavity preparation and filling materials also remain unsealed. Subsequently, because of residual bacteria on the cavity base, recurrent caries, leakage at the margin of fillings, or further operations, these processes may be exposed to bacteria which, if successfully established, can reach the pulp. Small-scale invasions by this route offer a possible explanation for persistent discomfort associated with fillings and for the acute inflammatory response which so often follows the death of a pulp under an unlined silicate filling.

The sum of the damage to odontoblasts and their processes from disease, accident, and operative procedures scarcely needs emphasizing. On the other hand, the readiness with which bacteria will invade necrotic processes which are exposed to the mouth and the implications of this have not been fully recognized.

SUMMARY

An experiment was designed to test the ability of bacteria to invade odontoblastic processes of non-vital dentin when it was exposed to the mouth. Crowns of freshly extracted premolars from which the occlusal enamel and pulps had been removed were worn in the mouth for varying periods. Subsequent examination of Gram-stained sections revealed that, in the brief period of 3 weeks, bacteria had invaded odontoblastic processes and reached the pulp chamber.

The conclusion was reached, therefore, that if odontoblastic processes succumb following death of the pulp or peripheral injury and are exposed to the mouth, they are liable to invasion by bacteria present. This conclusion is discussed in relation to (1) the presence of bacteria in advance of decalcification under a carious lesion; (2) infection of pulps of teeth which die following a blow; and (3) odontoblastic processes injured by operative procedures and subsequently exposed to bacteria.

The author is grateful to Professor F. R. Shroff for use of facilities, Professor G. N. Davies for the statistical analysis, and to Mr. C. Kennelly for preparation of histological sections.

REFERENCES

1. Rushton, M. A. The Exposed Dentine of Lower Incisor Teeth, *Brit. D. J.*, **84**:91, 1948.
2. Hodson, J. J. A Histopathological Study of the Bacterial Plaque in Relation to the Destruction of Enamel, Dentine and Bone with Special Reference to Dental Caries, *Proc. Roy. Soc. Med.*, **48**:641, 1955.
3. Chirnside, I. M. The Bacteriological Status of Dentine around Infected Pulp Canals, *N.Z. D. J.*, **54**:173, 1958.
4. Boyle, P. E. *Kronfeld's Histopathology of the Teeth,* p. 141. Philadelphia: Lea & Febiger, 1955.
5. Zander, H. A. Use of Silver Nitrate in the Treatment of Caries, *J.A.D.A.*, **28**:1260, 1941.
6. MacGregor, A., Marsland, E. A., and Batty, I. Experimental Studies of Dental Caries, *Brit. D. J.*, **101**:230, 1956.
7. Dorfman, A., Stephan, R. M., and Muntz, J. A. In Vitro Studies on Sterilisation of Carious Dentine, *J.A.D.A.*, **30**:1901, 1943.
8. Sognnaes, R. F., and Wislocki, G. B. Histological Observations on Enamel and Dentine Undergoing Carious Destruction, *J. Oral Surg., Oral Med., Oral Path.*, **3**:1283, 1950.
9. Fish, E. W. Lesions of the Dentine and Their Significance in the Production of Dental Caries, *J.A.D.A.*, **17**:992, 1930.

10. MACDONALD, J. B., HARE, G. C., and WOOD, A. W. S. The Bacteriological Status of the Pulp Chambers in Intact Teeth Found to be Non-Vital Following Trauma, *J. Oral Surg., Oral Med., Oral Path.*, **10**:318, 1957.
11. CHIRNSIDE, I. M. A Bacteriological and Histological Study of Traumatised Teeth, *N.Z. D. J.*, **53**:176, 1957.
12. MANLEY, E. B., and HARDWICK, J. L. Caries of the Enamel, *Brit. D. J.*, **91**:36, 1951.
13. FISH, E. W. *An Experimental Investigation of Enamel, Dentine and the Dental Pulp*, p. 95. London: John Bale Sons & Danielsson, Ltd., 1933.

Periodic Acid–Schiff Reactive Components of Human Gingiva

S. D. SCHULTZ-HAUDT, S. PAUS, and S. ASSEV
Department of Physiology, Dental Faculty, University of Oslo, Norway

Histochemical observations indicate the presence of extracellular substances in the connective tissue of human gingiva which are stained by the periodic acid–Schiff (PAS) procedure.[1–4] There is evidence to the effect that such PAS-positive substances contain polysaccharides which are different in composition from acid mucopolysaccharides.[5–7] Furthermore, it is not probable that the acid mucopolysaccharides of gingival[6] or other connective tissues stain by the PAS reaction.[7]

The present article deals with the isolation from human gingiva of two different polysaccharide-protein complexes which are both PAS-positive but neither of which contain acid mucopolysaccharides.

MATERIALS AND METHODS

Tissue material.—Gingival tissues were obtained from patients who were subjected to gingivectomy as part of the treatment of their periodontal lesions. These tissues were chronically inflamed. In addition, clinically normal tissues were excised from a number of patients at the time of tooth extractions. The tissue pieces, immediately upon removal, were rinsed in tap water and subsequently immersed in cold acetone (4° C.). The tissues obtained were pooled. When sufficient amounts had been collected, the pieces were finely divided by scissors, dried at room temperature, and next ground to a fine powder in a Culatti mill. The powder was treated with acetone, then with ether, and dried at room temperature.

Tissue extraction.—Two portions of 2 and 3.5 gm., respectively, of dry tissue powder were extracted in the manner shown in the chart on page 142.[8]

Paper electrophoresis.—This was carried out in the LKB apparatus.* The papers used were either the standard strips provided by the LKB company† or strips of the same dimensions (40 × 410 mm.) prepared from Whatman No. 1 filter paper. The buffer employed was the Michaelis veronal buffer of an ionic concentration of 0.125 and of pH 8.6.

Prior to electrophoresis, the paper strips were moistened with buffer and placed in the cassette. After equilibration for an hour, 50 μl. of solutions of the fractions in the buffer, containing about 1.5 mg. of dissolved material, were applied to each moist paper strip along a line 11.5 cm. from the cathode end of the strips. Thirty minutes

The investigations are supported by a grant from the Norwegian Research Council for Science and the Humanities.

Received for publication May 26, 1960.

* LKB-produkter, Stockholm, Sweden.

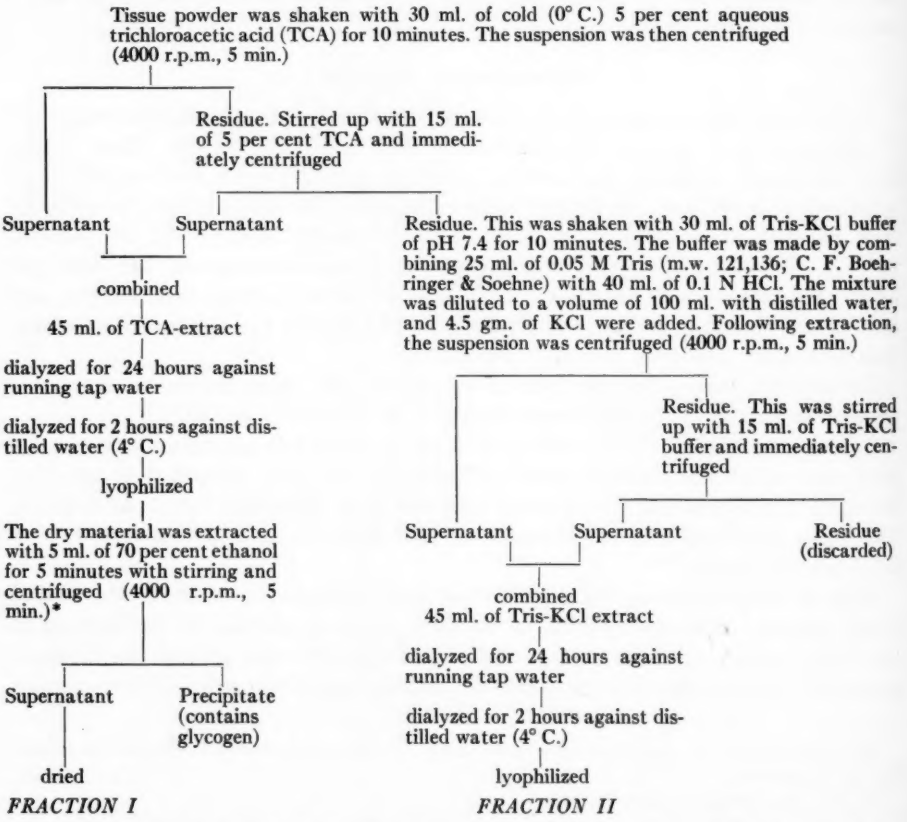
† Carl Schleicher & Schüll No. 2043 B filter paper.

after the application of the samples the current was connected and adjusted to 6 mA. Electrophoresis was carried out for 18 hours at room temperature. Following electrophoresis, the strips were dried in an oven at 110° C.

One strip from each electrophoresis series was divided lengthwise, and the ribbons were stained. The staining reactions included the amidoschwartz procedure for the visualization of proteins,[9] the periodic acid–Schiff technique according to Björnesjö,[10] and the cetavlon-bromocreosol purple method for the demonstration of acidic polysaccharides as described by Bera, Foster, and Stacey.[11]

From unstained strips of each series the areas corresponding to PAS-positive zones of the stained strips were sectioned out. The sections were eluted in the manner described by Rienits.[12] The eluates were dialyzed for 24 hours against running tap water and subsequently lyophilized.

Paper chromatography.—Samples of the two fractions (at least 2 mg.) and dried eluates obtained after paper electrophoresis were hydrolyzed with 2 ml. of 0.5 N HCl for 2 hours at 110° C. in sealed test tubes. Some of the hydrolysates were lyophilized directly, others following treatment with ion-exchange resins. In the latter case they


Tissue powder was shaken with 30 ml. of cold (0° C.) 5 per cent aqueous trichloroacetic acid (TCA) for 10 minutes. The suspension was then centrifuged (4000 r.p.m., 5 min.)
Residue. Stirred up with 15 ml. of 5 per cent TCA and immediately centrifuged
Supernatant
Supernatant
combined
45 ml. of TCA-extract
dialyzed for 24 hours against running tap water
dialyzed for 2 hours against distilled water (4° C.)
lyophilized
The dry material was extracted with 5 ml. of 70 per cent ethanol for 5 minutes with stirring and centrifuged (4000 r.p.m., 5 min.)*
Supernatant
Precipitate (contains glycogen)
dried
FRACTION I
Residue. This was shaken with 30 ml. of Tris-KCl buffer of pH 7.4 for 10 minutes. The buffer was made by combining 25 ml. of 0.05 M Tris (m.w. 121,136; C. F. Boehringer & Soehne) with 40 ml. of 0.1 N HCl. The mixture was diluted to a volume of 100 ml. with distilled water, and 4.5 gm. of KCl were added. Following extraction, the suspension was centrifuged (4000 r.p.m., 5 min.)
Residue. This was stirred up with 15 ml. of Tris-KCl buffer and immediately centrifuged
Supernatant
Supernatant
Residue (discarded)
combined
45 ml. of Tris-KCl extract
dialyzed for 24 hours against running tap water
dialyzed for 2 hours against distilled water (4° C.)
lyophilized
FRACTION II

* This extraction was introduced in order to remove glycogen, which was found to be a contaminant of Fraction I (see Discussion).

were shaken for 20 minutes with 50 mg. of Dowex 50 H^+, X12 (200–400 mesh). The suspension was then filtered with suction through filter paper on a small Buchner funnel. The resin was thoroughly washed with distilled water, and the washings added to the filtrate. The solution was next shaken with 50 mg. of Dowex 2 OH^-, X-8 (200–400 mesh) for 20 minutes and the above procedure repeated. The filtrates were finally lyophilized.

The dry hydrolysates were dissolved in 100 μl. of distilled water and portions of 50 and 25 μl. were spotted onto Whatman No. 1 filter paper. Paper chromatography was carried out either ascending or descending. In the ascending systems the papers were irrigated for 17 hours with ethyl acetate/pyridine/water (2/1/2)[13] or with *n*-butanol/pyridine/water.[14] Descending chromatography was carried out for 22 hours with *n*-butanol/pyridine/water[14] or with *n*-butanol/acetic acid/water (4/1/5) as the irrigation fluids. Following development, the papers were dried at room temperature for 2 hours. Sugar spots were demonstrated by spraying with aniline oxalate[15] or with silver nitrate as described by Stoffyn and Jeanloz.[16]

Hydroxyproline determinations.—When the contents of hydroxyproline were to be determined in samples of the fractions or in eluates from the paper sections, they were hydrolyzed with 2 ml. of 4 N HCl for 17 hours at 110° C. in sealed test tubes. The hydrolysates were dried by distillation at reduced pressure. The dry hydrolysates were dissolved in suitable amounts of water, and the hydroxyproline was determined in aliquots of the solutions according to the method of Martin and Axelrod.[17]

RESULTS

Tissue extraction.—The yields of the two fractions obtained in the manner described are listed in Table 1.

Paper electrophoresis.—When glycogen had not been removed by ethanol extraction of the TCA-extract, the amidoschwartz reaction revealed that Fraction I, upon electrophoresis, separated into three protein components. Of these, one reacted with the PAS stain as well, whereas the remaining two were PAS-negative, as shown in Figure 1.

TABLE 1

Amounts of Dry Tissue Powder Extracted (Gm.)	Amounts of Fraction I (TCA-Extract) (Mg.)	Amounts of Fraction II (Tris-KCl Extract) (Mg.)
2.0	5.2*	24.8
3.5	6.0	40.0

* Glycogen not removed by ethanol extraction.

When the TCA-extract had been treated with 70 per cent ethanol, the slowest-moving protein component no longer appeared on the paper strips following electrophoresis. The remaining two migrated and stained as before.

Upon electrophoresis, Fraction II separated into four protein components. The fastest- and the slowest-moving ones were well defined, whereas the components

located between the two were not as sharply outlined. Of the four, two reacted with the PAS stain as well, as shown in Figure 2.

The slowest-migrating component of Fraction II corresponded to the PAS-positive constituent of Fraction I. It could be removed from Fraction II by thorough rinsing of the tissue with 5 per cent trichloroacetic acid prior to the extraction with the Tris-KCl buffer.

Neither PAS-positive component of each fraction stained on the paper strips by the cetavlon-bromocreosol purple method, in contrast to hyaluronate and chondroitin

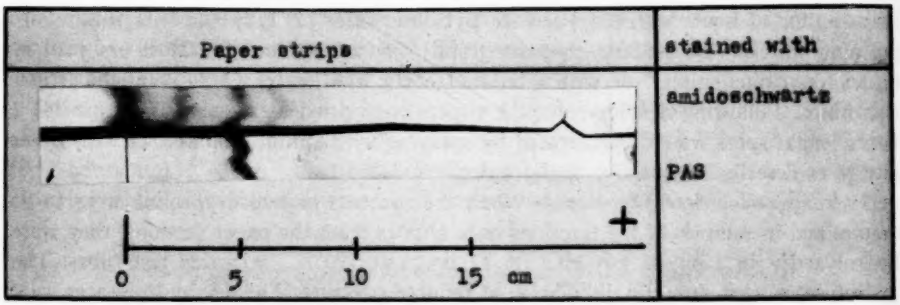


FIG. 1.—Fraction I stained with amidoschwartz and PAS

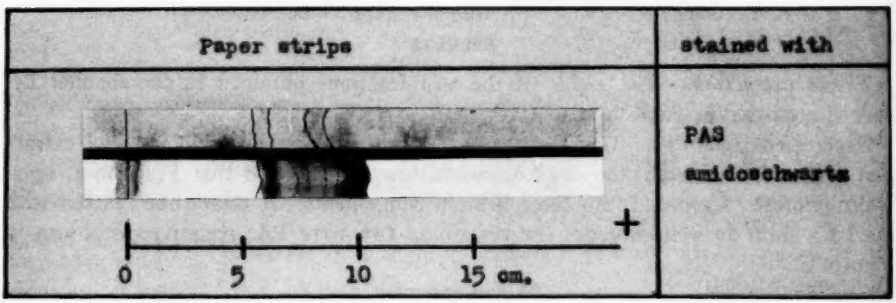


FIG. 2.—Fraction II stained with amidoschwartz and PAS

sulfate. Furthermore, hyaluronate migrated slightly faster toward the anode than did the PAS-positive component of Fraction II under identical experimental conditions. Chondroitin sulfate was, following electrophoresis, located toward the anode edge of the paper.

Paper chromatography.—The following sugars were found to be present in hydrolysates of the two fractions or of the eluates from the paper of their PAS-positive components:

Fraction I (TCA-Extract)	Fraction II (Tris/KCl-Extract)
Glucuronic acid	Glucuronic acid
Unknown	Glucosamine
Glucosamine	Galactose
Glucose	Mannose
Mannose	(Fucose)
Ribose	Ribose

The "unknown" component of Fraction I may be a heptose.[8] Spots representing fucose in hydrolysates of Fraction II were uncertain. Glucuronic acid and glucosamine were demonstrated in hydolysates which had not been treated with ion-exchange resins following hydrolysis.

Hydroxyproline determinations.—These revealed that the polysaccharide of Fraction I was associated with a protein containing hydroxyproline. Measurements on a hydrolyzed eluate corresponding to the PAS-positive zone of one paper strip gave readings corresponding to the presence of 20 μg. of hydroxyproline when about 1.5 mg. of this fraction (containing glycogen) had been applied to the paper. In another case, when glycogen had been removed by ethanol extraction and about 2 mg. of the fraction had been applied to the paper, there were 48 μg. of hydroxyproline present in the hydrolyzed eluate from one paper strip. Similar determinations on Fraction II were negative.

DISCUSSION

A variety of extraction methods was tested.[8] The one described above was preferred because it permitted the isolation in successive steps of two different polysaccharide-protein complexes from connective tissues of skin and gingiva.

The migration of the polysaccharide-protein component of Fraction I was apparently due to electro-osmosis. This assumption was based on tests in which glycogen was subjected to the same electrophoretic conditions as were the obtained fractions. Staining of the strips by means of the PAS reagents revealed that the glycogen was located at a distance from the starting line that corresponded entirely to the position of the PAS-positive component of Fraction I. This observation led to the conclusion that this component was contaminated by glycogen. The glycogen, however, could be removed by extracting the dried fraction with 70 per cent ethanol, as described above. Since the PAS-positive component migrated in a manner identical with the displacement of glycogen toward the anode, it was assumed that it did not possess a net negative charge. It should be added that, by the ethanol extraction, small amounts of a protein containing hydroxyproline were precipitated as well.

The PAS-positive component of Fraction II, however, appeared to carry a small net negative charge, since it became displaced farther toward the anode end of the paper. The rate of migration was slightly slower than that of a hyaluronate-protein complex tested in the same manner. This latter complex, in turn, migrated at a slower rate than did a chondroitin sulfate–protein complex subjected to identical experimental conditions.

As previously pointed out, neither the hyaluronate nor the chondroitin sulfate tested reacted by the PAS procedure as applied to paper strips. This supports the observations of Leblond *et al.*[7] that these acid mucopolysaccharides are not responsible for the reaction of the connective tissue ground substance with the periodic acid–Schiff stain. The experiments here presented suggest that the polysaccharide moiety of the two polysaccharide-protein fractions obtained represent the PAS-positive components of the gingival connective tissue ground substance.

As was pointed out above, the PAS-positive component of Fraction I was found to be a polysaccharide associated with a protein containing hydroxyproline. Thus and because the complex is water-soluble, the protein moiety may be some type of

soluble collagen.[18–20] Because of the scarcity of source material available, it was not determined which of the known soluble varieties—if any of them—the fraction contained. It has been established, however, that human gingiva contains relatively fair amounts of soluble collagens,[21] part of which may represent stages in the formation of collagenous fibers. It may be noted in this connection that accumulation of PAS-positive material in the ground substance occurs in wound healing, preceding the deposition of collagenous fibers.[22] Much of the tissue material extracted in the present experiments was chronically inflamed. Chronic gingivitis proceeds slowly, the process involving intermittent periods of tissue repair. However, even clinically normal gingiva regularly contains histologically demonstrable areas of chronic inflammation. In a general way, therefore, most of the source material in these experiments would be expected to have existed in some stage of tissue repair. Thus the polysaccharide-protein component of Fraction I may represent a precursor of the insoluble collagenous fibers.

SUMMARY

An extraction method has been developed which permitted the isolation from human gingiva of two polysaccharide-protein complexes with different electrophoretic mobilities. Both complexes reacted with the periodic acid–Schiff procedure as employed in the experiments, whereas neither hyaluronate nor chondroitin sulfate stained.

The polysaccharide moiety of one of the complexes was found to contain glucuronic acid, glucosamine, galactose, glucose, mannose, and ribose and, in addition, a component tentatively described as a heptose. This heteropolysaccharide was associated with a protein containing hydroxyproline. It was suggested that the protein part of the complex was a soluble collagen and that the complex as a whole may represent some stage in the synthesis of collagenous fibers.

The polysaccharide part of the second complex contained glucuronic acid, glucosamine, galactose, glucose, mannose, ribose, and possibly fucose. The nature of the protein apparently associated with this heteropolysaccharide has not yet been established.

REFERENCES

1. McManus, J. F. A. Histological Demonstration of Mucin after Periodic Acid, *Nature*, **158**: 202, 1946.
2. Hotchkiss, R. D. A Microchemical Reaction Resulting in the Staining of Polysaccharide Structures in Fixed Tissue Preparations, *Arch. Biochem.*, **16**:131, 1948.
3. Wislocki, G. B., and Sognnaes, R. F. Histochemical Reactions of Normal Teeth, *Am. J. Anat.*, **87**:239, 1950.
4. Turesky, S., Glickman, I., and Litwin, T. A Histochemical Evaluation of Normal and Inflamed Human Gingiva, *J. D. Res.*, **30**:792, 1951.
5. Dische, Z., Danilczenko, A., and Zelmenis, G. The Neutral Heteropolysaccharides in Connective Tissue. In: *Chemistry and Biology of Mucopolysaccharides*. (A Ciba Foundation Symposium.) London: J. & A. Churchill, Ltd., 1958.
6. Schultz-Haudt, S. D. *Observations on the Acid Mucopolysaccharides of Human Gingiva*. Oslo: Oslo University Press, 1957.
7. Leblond, C. P., Gregg, R. E., and Eidinger, D. Presence of Carbohydrates with Free 1,2-Glycol Groups in Sites Stained by the Periodic Acid–Schiff Technique, *J. Histochem. Cytochem.*, **5**:445, 1957.
8. Eeg-Larsen, N. L., and Schultz-Haudt, S. D. Heteropolysaccharides of Animal Skin, *Acta Physiol. Scand.*, **50**:139, 1960 (Suppl. 175).

9. GRASSMANN, W. Quoted in: R. J. BLOCK, E. L. DURRUM, and G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*. New York: Academic Press, Inc., 1955.
10. BJÖRNESJÖ, K. B. Staining of Protein-bound Serum Polysaccharides in Electrophoresis Strips, *Scand. J. Clin., Lab. Invest.*, **7**:153, 1955.
11. BERA, B. C., FOSTER, A. B., and STACEY, M. Observations on the Properties of Cetyltrimethylammonium Salts of Some Acidic Polysaccharides, *J. Chem. Soc.*, p. 3788, 1955.
12. RIENITS, K. G. The Electrophoresis of Acid Mucopolysaccharides on Filter Paper, *Biochem. J.*, **53**:79, 1953.
13. JERMYN, M. A., and ISHERWOOD, F. A. Improved Separation of Sugars on the Paper Partition Chromatogram, *Biochem. J.*, **44**:402, 1949.
14. CHARGAFF, E., LEVINE, C., and GREEN, C. Techniques for the Demonstration by Chromatography of Nitrogenous Lipide Constituents, Sulfur-containing Amino Acids, and Reducing Sugars, *J. Biol. Chem.*, **175**:67, 1948.
15. HORROCKS, R. H. Paper Partition Chromatography of Reducing Sugars with Benzidine as a Spraying Reagent, *Nature*, **164**:444, 1949.
16. STOFFYN, P. J., and JEANLOZ, R. W. Identification of Amino Sugars by Paper Chromatography, *Arch. Biochem. Biophys.*, **52**:373, 1954.
17. MARTIN, C. J., and AXELROD, A. E. A Modified Method for Determination of Hydroxyproline, *Proc. Soc. Exptl. Biol. Med.*, **83**:461, 1953.
18. HARKNESS, R. D., MARKO, A. M., MUIR, H. M., and NEUBERGER, A. The Metabolism of Collagen and Other Proteins of the Skin of Rabbits, *Biochem. J.*, **56**:558, 1954.
19. JACKSON, D. S. Connective Tissue Growth Stimulated by Carrageenin. 1. The Formation and Removal of Collagen, *Biochem. J.*, **65**:277, 1957.
20. GREEN, N. M., and LOWTHER, D. A. Formation of Collagen Hydroxyproline *in vitro*, *Biochem. J.*, **71**:55, 1959.
21. SCHULTZ-HAUDT, S. D., and AAS, E. Observations on the Status of Collagen in Human Gingiva, *Arch. Oral Biol.*, **2**:131, 1960.
22. GERSH, I., and CATCHPOLE, H. R. The Organization of Ground Substance and Basement Membrane and Its Significance in Tissue Injury, Disease, and Growth, *Am. J. Anat.*, **85**:457, 1949.

A Centrifugal Technique of Measuring Food Retention

DONALD J. BECK and BASIL G. BIBBY
Eastman Dental Dispensary, Rochester, New York

Since the retention of carbohydrate foods on the teeth is accepted as a determinant of caries activity, it is logical to assume that there will be a direct relationship between the extent to which carbohydrate foods adhere to the teeth and their relative cariogenicity. Therefore, if foods could be rated according to their retentivity to the teeth, there might be reason for excluding some carbohydrates but allowing others in diets designed to control caries.

This concept led Bibby, Goldberg, and Chen[1] to determine "decalcification potentials" for 105 foods from experiments which combined measurements of the amount of food retained around the teeth following mastication and the amount of acid formed on salivary fermentation of that food.

Bibby *et al.*[1] measured food retention by means of a carbohydrate analysis of rinsings recovered from the mouth of a test subject who had chewed the food. Their technique for measuring food retention was subject to several inaccuracies, the most important of which was that the value obtained included the food retained on the soft tissues of the mouth as well as that retained on the teeth. Although this was overcome by Ludwig and Bibby's[2] modification, the method was still subject to considerable person-to-person and day-to-day variations.

In view of these uncontrollable limitations in the in vivo technique, it was considered desirable to develop an in vitro method of measuring the retention of foods to tooth surfaces. Such an in vitro technique could be used to derive cariogenic ratings of foods, and it could also be employed to analyze the factors in the composition or preparation of foods which influenced their retentivity. This information might in turn make it possible to modify the formulation of candies and other highly cariogenic foods to reduce their cariogenicity without affecting their taste appeal.

This paper reports the development of such an in vitro technique which gives a fairly wide spread in numerical values for different foods and which rates the foods in much the same order of retentivity as had been obtained with earlier, intra-oral recovery techniques.

EXPERIMENTAL METHODS AND RESULTS

Development of centrifugal method.—After preliminary experimentation with apparatus designed to imitate masticatory movements, it was decided that centrifugal force was the most easily controlled force that could be used to dislodge test foodstuffs

This investigation was supported in part by Research Grant D-607 from the N.I.H., U.S. Public Health Service.

Received for publication May 27, 1960.

from tooth surfaces. Accordingly, an apparatus was designed in which the amount of food/saliva mixtures left adhering to teeth mounted in the caps of centrifuge tubes following controlled centrifugation (which tended to throw most of the mixture from around the teeth where it had originally been placed) could be determined by a carbohydrate analysis method, similar to that used by Bibby *et al.*[1] for their intra-oral recovery technique. During the development of this procedure the following tests, standardizing the temperature conditions and the speed and period of centrifugation, were conducted.

Temperature conditions.—Early tests indicated that the temperature of the samples, which differed according to the time of standing after collection, appeared to have a significant effect on the retention results obtained. To control this variable and thereby improve the reproducibility of results, it seemed desirable to incubate samples at 37° C. to bring them to a uniform temperature before centrifugation. To investigate

TABLE 1

EFFECT OF INCREASING PERIOD FOR WHICH FOUR FOOD/SALIVA MIXTURES WERE STORED IN A 37° C. INCUBATOR PRIOR TO CENTRIFUGATION UPON CENTRIFUGAL RETENTION INDEX

Food	Centrifugal Retention Index (Mg.) and Percentage Decrease			
	Period in Incubator Prior to Centrifugation (Minutes)			
	0	10	20	30
Chocolate	3,157	2,017 (36%)	1,612 (49%)	595 (81%)
Caramel	551	300 (46%)	290 (47%)	228 (59%)
Corn flakes	194	123 (37%)	101 (48%)	75 (61%)
Arrowroot biscuit	179	145 (19%)	113 (37%)	73 (59%)

this point, four foods—chocolate, caramel, corn flakes, and arrowroot biscuit—were chewed by one of us (D.J.B.) and ejected to produce test samples. These were loaded on the centrifuge caps and left at room temperature for 15 minutes to allow the food to cool from mouth temperature. Following this, the samples of each food were placed in a 37° C. incubator for either 0, 10, 20, or 30 minutes, centrifuged, and carried through the usual test procedures.

Table 1 shows the results of this experiment. In all cases, with increasing temperatures of the samples (i.e., increasing periods of incubation prior to centrifugation), decreasing values for the centrifugal retention indexes (i.e., the amount of food, in milligrams, left adhering to the teeth) were recorded. As might be expected, this effect was most marked with chocolate, which at room temperature is solid but which at 37° C. is semiliquid. The considerable variations recorded even with corn flakes and arrowroot biscuit were more surprising, for an inspection of these mixtures showed little change in their consistency with increasing temperatures. The possibility that some drying-out of the samples or amylolytic activity occurred during the period they were stored in the incubator and that this was affecting the results needs to be considered.

Speed and period of centrifugation.—To arrive at the optimum speed and time of centrifugation a number of preliminary tests were run. Two such experimental series typical of this type are presented, in which the only variations were in the speed and period of centrifugation. The test food was 20 gm. of shortbread blended for 1 minute in a heavy-duty electric mortar* with 7.5 ml. of saliva from the same donor to give a mixture approximately the consistency of a natural bolus. (It was found in these early trials that reproducibility could be greatly increased by the use of artificial blending of food and saliva rather than natural chewing.) The first experiment employed a speed of 500 rpm, which submitted the food to a dislodging, relative centrifugal force† of 25 gravities. Duplicate tests at this rotational speed were run for 5, 10, 20, and 30 minutes.

In the second series duplicate runs of 1, 2, 3, 5, 10, 20, and 30 minutes were made at a rotational speed of 900 rpm, giving a relative centrifugal force of 82 gravities.

TABLE 2

VARIATIONS IN CENTRIFUGAL RETENTION INDEX OF SHORTBREAD WITH INCREASING PERIODS OF CENTRIFUGATION AT TWO ROTATIONAL SPEEDS

Period of Centrifugation (Minutes)	Centrifugal Retention Index (Mg.)					
	Low Relative Centrifugal Force (R.C.F.: 25 g; rpm: 500)			High Relative Centrifugal Force (R.C.F.: 82 g; rpm: 900)		
	Trial 1	Trial 2	Mean	Trial 1	Trial 2	Mean
1	.....	.....	.....	1,243	1,189	1,216
2	.....	.....	.....	397	321	359
3	.....	.....	.....	282	211	247
5	589	450	520	181	198	190
10	638	435	537	191	187	189
20	724	708	716	208	152	180
30	663	539	601	170	184	177

In both cases it required 4 minutes 30 seconds to attain the steady speed of 500 or 900 rpm.

Table 2 and Figure 1 show that at the low rotational speed of 500 rpm there was poor reproducibility, and no coherent pattern of results was obtained.

More satisfactory patterns of values were given at the high rotational speed. The bulk of the food was apparently thrown off the teeth during the first 5 minutes of centrifugation, and prolonging the centrifugation beyond this period had only a minimum effect. To obtain values well out on the flat part of the curve shown in Figure 1, where minor variations in the period of centrifugation would have a negligible effect

* Crescent Heavy Duty Wig-L-Bug No. 6, Crescent Dental Manufacturing Co., Chicago 23, Illinois.

† Relative centrifugal force (see ref. 3) is determined from the following equation:

$$\text{R.C.F.} = 0.0000118 \times r \times N^2,$$

where: R.C.F. = Relative centrifugal force (gravities), r = rotating radius (cm.), and N = rotating speed (rpm). With the equipment used in these trials, the rotating radius was 9 cm.

on the value obtained and where some degree of reproducibility was assured, 900 rpm for 15 minutes was chosen as the standard procedure.

The standard centrifugal retention method.—On the basis of these and related standardization procedures the following standard method was derived:

The basic apparatus for the centrifugal retention technique is shown in Figure 2. The crowns of four extracted, sound, human molar teeth were mounted in a small acrylic base which was attached by two bolts to an acrylic cap made to fit a 2½-inch centrifuge tube. Four such caps were made, two employing upper molar teeth and two employing lower molar teeth.

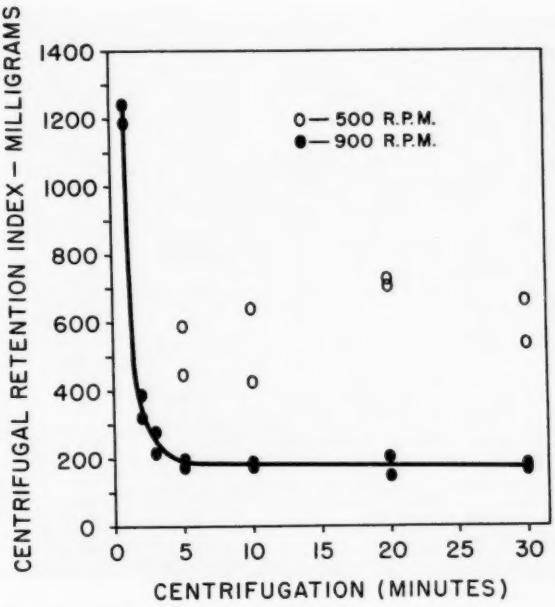


FIG. 1.—Effect of varying periods of centrifugation upon the centrifugal retention index of shortbread. Two series of trials employing 500 and 900 rpm.

The food under test was prepared by artificial blending with saliva, using 60-ml. polystyrene vials and a polyethylene pestle 4 cm. long and 1 cm. in diameter.

A gross excess of the food/saliva mixture was coated on the teeth after prior painting of the tooth surfaces with saliva. During the application of the food to the teeth, which was carried out with a finger moistened in saliva, care was taken to completely fill the occlusal fissures and proximal spaces.

In an effort to standardize the temperature conditions of all tests, the centrifuge equipment—teeth, caps, glass tubes, and metal caps—was stored at all times in an incubator maintained at 37° C., and, after the application of the prepared food to the teeth, the caps and teeth were returned to the incubator for 30 minutes to increase the temperature of the samples to something approaching body temperature.

Following this period in the incubator, the loaded caps and tubes were transferred to a centrifuge* and centrifuged for a 15-minute period, accelerating to a maximum

* International Model U Centrifuge.

speed of 900 rpm. At the end of the controlled run, the centrifuge was automatically cut off and, within 3 seconds following this cutoff, was braked to a complete stop.

After centrifugation the bulk of the food had been thrown from the teeth to the bottom of the centrifuge tubes. To determine the amount of food left adhering to the teeth, the tooth segments were removed from the caps and washed in standard volume of distilled water in a mechanical shaker. The food content of these washings was determined by blending them in an electric blender,* diluting to the required carbohydrate concentration range, and subjecting them to a modified anthrone colorimetric technique (Ludwig and Goldberg,[4] Beck and Bibby[5]) for carbohydrate analysis. From

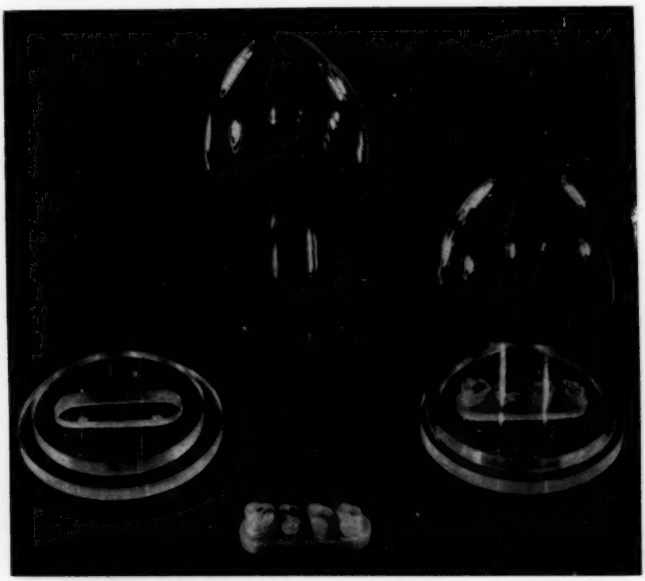


FIG. 2.—Basic equipment for the centrifugal retention technique. Four human molar teeth, mounted in a block of acrylic resin, attached by two bolts to the cap of a centrifuge tube of internal diameter 2½ inches. Four such teeth-cap-tube assemblies were employed in each test.

the carbohydrate values so obtained and the previously determined per cent carbohydrate content of the test foodstuff, it was possible to determine the amount of food that was adhering to the four tooth segments. To check the reproducibility of values obtained by the technique, duplicate trials were run in most cases. The mean of the amounts of food retained in the two trials, in milligrams, was taken to be the centrifugal retention index of the food.

Effect of variations in saliva content of test samples.—One of the first problems related to food retention investigated with this standardized procedure was that of the effect of varying composition of the food/saliva mixture. For this purpose, samples composed of 20 gm. of corn flakes, blended with 15, 20, 25, 30, 35, 40, 45, or 50 ml. of saliva, were carried through duplicate trials.

The results in Table 3 and Figure 3 show that the amount of saliva in the mixture

* Waring Blendor.

had a significant effect on the retention values obtained. From a very dry mix to one of approximately the consistency of a natural bolus (30 ml. of saliva, 20 gm. of corn flakes), increasing the amount of saliva produced a sharp decrease in the retention values obtained. However, beyond this point, as the mixture became more and more liquid, the retention values gradually increased. The reason for this latter effect is not known.

Application of centrifugal retention technique to twelve representative foods.—Two series of tests were made. The first was conducted relatively early in the development of the centrifugal retention technique, when test food samples were produced by natural chewing instead of artificial blending, and the experimental centrifuge caps

TABLE 3

EFFECT OF INCREASING VOLUME OF SALIVA BLENDED WITH 20 GM. OF CORN FLAKES ON CENTRIFUGAL RETENTION INDEX; DUPLICATE TRIALS WITH EACH MIXTURE

VOLUME OF SALIVA IN MIXTURE (ML.)	CENTRIFUGAL RETENTION INDEX (MG.)		
	Trial 1	Trial 2	Mean
15	359	229	294
20	122	113	118
25	59	51	55
30	51	48	50
35	66	66	66
40	69	81	75
45	87	83	85
50	105	101	103

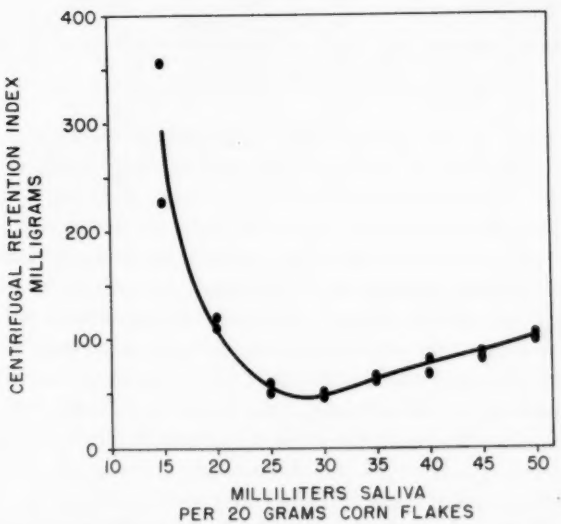


FIG. 3.—Effect of varying amounts of saliva blended with constant amounts of corn flakes upon the centrifugal retention index.

carried just two teeth each instead of the four teeth used later. This series had as one objective a comparison of the centrifugal results with intra-oral retention figures obtained from in vivo tests made by the technique of Ludwig and Bibby.[2] The results are presented in Table 4 in the form of a comparison of the centrifugal retention technique and the intra-oral recovery technique. Some correlation in the order of retentivity of the foods in the two lists is evident, but the correlation is not perfect.

The second series of tests used the standardized procedure, with artificial blending of test samples and centrifuge caps carrying four teeth each. To provide food mixtures of suitable consistency, a practical, if somewhat unscientific, procedure was followed. By trial and error, the proportions of food and saliva were determined for each food which, on blending, produced a mix that appeared to the investigators to be approxi-

TABLE 4

TWELVE FOODS LISTED IN ORDER OF RETENTIVITY AS DETERMINED BY INTRA-ORAL RECOVERY TECHNIQUE AND CENTRIFUGAL RETENTION TECHNIQUE (USING NATURALLY CHEWED TEST SAMPLES AND TWO-TOOTH CENTRIFUGE CAPS)

INTRA-ORAL RECOVERY TECHNIQUE		CENTRIFUGAL RETENTION TECHNIQUE	
Foodstuff	Food Retained (Mg.)	Foodstuff	Food Retained (Mg.)
Caramel	127	Caramel	525
Prune (dried)	104	Milk chocolate	308
White bread and peanut butter	102	White bread	159
Apple	86	White bread and peanut butter	140
Milk chocolate	85	Prune (dried)	128
White bread	80	White bread and butter	126
Mound bar	77	Mound bar	124
Corn flakes	73	Shredded wheat and milk	117
White bread and butter	63	Apple	106
Pineapple (canned)	44	Corn flakes	68
Shredded wheat and milk	40	Pineapple (canned)	45
Puffed rice	37	Puffed rice	39

mately the consistency of the natural bolus obtained by chewing that food to the point of swallowing. This mix in each case was designated a "medium" mix. A "thin" mix was produced by adding sufficient additional saliva to the food so that the mixture would pour from one vessel to another. A "thick" mix was produced by blending each food with a minimum amount of saliva that would produce a dry, but homogeneous, mix. The actual proportions of saliva and food used to produce these three mixes for each food tested are recorded in Table 5. Each of the twelve foods was tested in this experiment in these three different consistencies, all thirty-six tests being duplicated.

The results of these tests, which are presented in Table 6 and Figure 4, reveal considerable variation in values obtained, both between different foods and between different consistencies of the same food. No consistent pattern can be found in the results. In some foods (puffed rice, the three candies, pineapple, bread and butter, and bread and peanut butter) the retention decreased consistently with increasing amounts of saliva in the mix, while in other foods (shredded wheat and milk, prune, and white bread) the "medium" mix was more retentive than either the very thick

mix or the thin one. With corn flakes and apples the "medium" mix was less retentive than either the "thick" mix or the "thin" one.

The over-all retention values for the candies tended, as one might expect, to be high, with particularly high values (six or more times higher than any other food) being recorded with the "thick" and "medium" mixes of chocolate. However, increasing the amount of saliva, to produce a "thin" mix, produced a tremendous drop in the retention of this food. Surprisingly high retention values were recorded for apple and surprisingly low values for dried prune. The addition of butter or peanut butter

TABLE 5

PROPORTIONS OF SALIVA IN FOOD/SALIVA MIXTURES USED IN CENTRIFUGAL RETENTION EXPERIMENTS RECORDED IN TABLE 6

FOODSTUFF	ML. SALIVA PER 20 GM. FOODSTUFF		
	"Thick" Mix	"Medium" Mix	"Thin" Mix
Cereals:			
Corn flakes	15	30	50
Puffed rice	10	20	40
Shredded wheat and milk*	0	10	20
Candies:			
Mound bar	2	5	10
Milk chocolate	2	5	10
Caramel	1	3	10
Fruits:			
Apple	2	10	20
Pineapple (canned)	1	3	10
Prune (dried)	5	10	20
Bread:			
White bread	10	20	40
White bread and butter†	10	20	40
White bread and peanut butter‡	10	20	40

* Shredded wheat, 10 gm.; milk, 10 gm.
† White bread, 16 gm.; butter, 4 gm.
‡ White bread, 16 gm.; peanut butter, 4 gm.

to bread appeared to have a negligible effect on the retention, apart from a slight increase in retention when the "thick" mixes were used.

A comparison of results recorded for the twelve foods with the intra-oral recovery technique (Table 4) and the centrifugal retention technique in this later experiment (Table 6) is difficult, in that we obtained three values for each food with the latter technique. However, some degree of over-all correlation may be detected, but again it is not a perfect correlation. Some foods appeared to be relatively more retentive when measured by one technique than when measured by other.

DISCUSSION

The centrifugal retention method, on the basis of our findings, seems to give reproducible results under many test conditions and therefore may have some long-range

value in making some types of comparisons between foodstuffs. On the other hand, the results, although reproducible, were often widely divergent with varying experimental conditions, as is shown in the results obtained by the centrifugal technique using three different ratios of food and saliva mixes as test material (Table 6) and when these results are compared with those obtained with the intra-oral technique (Table 4). That such variability results from changes in the proportions of food and saliva and other influences, such as temperature (Table 1), indicates the complexity of factors bearing on our experimental results. Presumably, intra-oral retention is similarly influenced.

Among the factors influencing retention of food/saliva mixtures are such physical factors as adhesion, cohesion, viscosity, homogeneity, particle size, specific gravity,

TABLE 6

CENTRIFUGAL RETENTION INDEXES RECORDED IN DUPLICATE EXPERIMENTS USING THREE DIFFERENT CONSISTENCIES OF SALIVARY MIXTURES WITH EACH OF TWELVE FOODS

FOODSTUFF	CENTRIFUGAL RETENTION INDEX (MG.)		
	"Thick" Mix	"Medium" Mix	"Thin" Mix
Cereals:			
Corn flakes	359, 229 — 294	51, 48 — 50	105, 101 — 103
Puffed rice	180, 281 — 231	92, 113 — 103	97, 89 — 93
Shredded wheat and milk	76, 72 — 74	110, 116 — 113	85, 94 — 89
Candies:			
Mound bar	240, 313 — 277	246, 216 — 231	164, 126 — 145
Milk chocolate	3,119, 3,802 — 3,461	1,300, 2,327 — 1,814	46, 51 — 49
Caramel	566, 425 — 496	128, 112 — 120	66, 63 — 6
Fruits:			
Apple	422, 405 — 414	254, 244 — 249	244, 340 — 292
Pineapple (canned)	228, 244 — 236	213, 220 — 217	137, 147 — 142
Prune (dried)	99, 123 — 111	144, 156 — 150	95, 96 — 96
Bread:			
White bread	77, 81 — 79	132, 134 — 133	116, 111 — 114
White bread and butter	145, 156 — 151	124, 91 — 108	85, 72 — 79
White bread and peanut butter	126, 85 — 106	105, 104 — 105	92, 77 — 85

and water content. If it were possible to measure each of these physical factors separately, as Caldwell[6] is attempting to do, it might be easier to produce specific explanations for the variations in the results obtained, but, unfortunately, such findings would still be as difficult to correlate with the part that the particular foods played in the causation of dental caries as is our attempted measurement of over-all in vitro food retention.

As far as we can interpret the significance of the various physical factors in the results we obtained, it would seem that the cohesion of the mixtures was most important. This conclusion is based on the observation that at the end of centrifugation

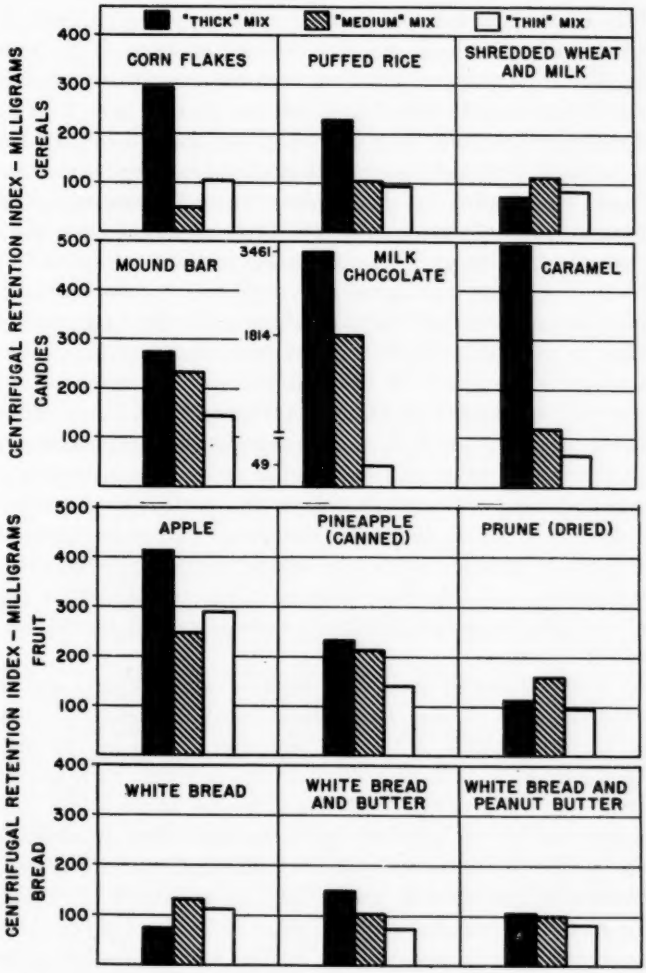


FIG. 4.—Centrifugal retention indexes of a group of foods tested in three different consistencies: (1) a "thick" mix, containing the minimum amount of saliva which would produce a homogeneous sample; (2) a "medium" mix, approximately the consistency of a natural bolus; (3) a "thin" mix, a semiliquid mix containing an excess of saliva. Each value on this diagram represents the mean of the results of two duplicate trials.

of most food samples there was still a thin layer of foodstuff on the tooth surfaces, indicating cohesive failure within the food, rather than adhesive failure between the food and tooth. Another factor which we feel may have had a bearing on the variability of the pattern of results obtained with different mixtures of the same food was the homogeneity of the food sample and the size of the particles within the mixture. More efficient mixing of the sample was apparent in some foods, such as shredded wheat, when there was more saliva in the mix. However, this conclusion is likewise supported only by visual impressions. The effect of the temperature of the food mixtures on their retention was quite marked (Table 1) and was probably principally the result of changes produced in the viscosity and cohesion of the test mixtures.

In view of the large number of factors involved in food retention and their interplay and variability from food to food and with different mixtures of the same food, it is not surprising that the results we obtained with the centrifugal retention technique were so widely divergent and showed no consistent pattern from food to food.

The retention figures obtained with varying proportions of saliva mixed with corn flakes (Table 3, Fig. 3), the most intensively studied food, are of interest in that they tend to emphasize the complexities of the subject of food retention and the difficulties of interpretation of food-retention results. The first part of the curve shown in Figure 3 shows the effect we anticipated, i.e., increasing amounts of saliva in the mixture decrease the retention value, and agrees very well with Caldwell's[6] findings for the pure adhesion of this food to tooth surfaces. However, in the latter part of this curve, the reverse effect is seen, i.e., retention values increasing with increasing amounts of saliva; therefore, some other factor in the food, in the saliva, in the mixture, or merely in our experimental technique must be more predominant in these wetter mixes. We can only theorize what this factor or factors may be. The explanation may be that the relative importance of adhesion and cohesion varies with increasing quantities of saliva or, as was suggested with shredded wheat, that particle size variation may occur with varying amounts of saliva. Increasing amounts of salivary amylase in the wetter mixtures may result in increased amylolytic activity during the 30 minutes' incubation prior to centrifugation, and this may affect retention, or differential evaporation of moisture from the different mixes during this same 30-minute incubation period may have an effect on retention.

To return to a comparison of the results obtained with the intra-oral tests and the centrifugal retention tests, it may be suggested that the relatively poor degree of correlation between the results of the two technqiues could be the result of either large experimental errors in both procedures or the fact that each procedure records different inherent properties of the foodstuffs. That the latter is the more likely explanation is supported by the relatively good reproducibility of results in duplicate controlled tests by both the intra-oral and the centrifugal procedure. Obviously, caution must therefore be exercised in interpreting the results of the centrifugal techniques into the mouth or relating it at this stage of its development to the complex in vivo factors contributing to dental caries. Further standardization of the procedure may produce results that can be related back to the mouth in the knowledge that they are a true representation of the degree to which foods are retained around the teeth after eating. Regardless, however, of the present or future usefulness of the centrif-

ugal retention method in its application to the cariogenic potential of foodstuffs, it does, in its present form, have application to related fields of study. It gives some information on the retention properties of foodstuffs, and the technique can safely be used for examining such problems related to food retention as the effectiveness of any agent incorporated in foods or coated on the teeth or any variation in foodstuff formulation designed to reduce food retention. For instance, it has been suggested[1] that the salt and fat content of foodstuffs influences their retention properties. This hypothesis could be simply tested by the centrifugal retention technique.

SUMMARY

In a search for a suitable in vitro technique for measuring the retention of foods to tooth surfaces, it was decided that centrifugal force offered the most convenient method of breaking food-tooth bonds and thereby gauging the retention properties of test foodstuffs. The technique consisted of placing a test food/saliva mixture around teeth mounted in the caps of centrifuge tubes. After controlled centrifugation, the teeth were removed from the caps, washed in standard volume of water, and the total carbohydrate in the washings determined, using a modified anthrone colorimetric technique. These values were used to determine the total amount of food adhering to the teeth after centrifugation, and this amount, in milligrams, was taken as the centrifugal retention index.

Increasing the temperature of the test sample was shown to decrease the values obtained for the centrifugal retention index. Variations in speed and period of centrifugation were demonstrated to influence the retention values obtained. At a low rotational speed, poor reproducibility of results was seen, but at a high rotational speed, good reproducibility with duplicate trials was apparent, and the centrifugal retention index decreased with increasing periods of centrifugation. Most of the food was thrown off the teeth during the first 5 minutes of centrifugation. Varying the proportions of saliva in the test mixture produced variations in the retention values, but this effect varied from food to food.

To afford a basis for comparison, twelve representative foods were tested using both the intra-oral recovery technique and the centrifugal retention technique. With the latter technique, three mixtures of each food, employing different proportions of saliva, were tested. Considerable variation in the retention properties of the different foods and of the different mixtures of each food was recorded. Correlation of results of the two techniques was only moderate. The various factors which may affect the retention of foodstuffs in the mouth and the centrifugal retention indexes and those factors which may have contributed to the complexity and difficulty of interpretation of results of the centrifugal retention technique and the relatively poor degree of correlation between results of the two retention techniques were discussed.

REFERENCES

1. Bibby, Basil G., Goldberg, Hyman J. V., and Chen, Eugene. Evaluation of Caries-producing Potentialities of Various Foodstuffs, *J.A.D.A.*, **42**:491, 1951.
2. Ludwig, T. G., and Bibby, B. G. Further Observations upon the Caries-producing Potentialities of Various Foodstuffs, *J. D. Res.*, **36**:61, 1957.

3. KENDRICK, ARTHUR. *The Application of Centrifugal Force* (Bulletin Y), p. 2. Boston, Mass.: International Equipment Co., 1957.
4. LUDWIG, THOMAS G., and GOLDBERG, HYMAN J. V. The Anthrone Method for the Determination of Carbohydrates in Foods and in Oral Rinsings, *J. D. Res.*, **35**:90, 1956.
5. BECK, DONALD J., and BIBBY, BASIL G. A Modified Anthrone Colorimetric Technic for Use in Investigations Related to the Cariogenicity of Foodstuffs, *J. D. Res.*, **40**:161, 1961.
6. CALDWELL, ROBERT C. A Method of Measuring the Adhesion of Foodstuffs to Tooth Surfaces, *J. D. Res.*, **38**:188, 1959.

A Modified Anthrone Colorimetric Technique for Use in Investigations Related to the Cariogenicity of Foodstuffs

DONALD J. BECK and BASIL G. BIBBY
Eastman Dental Dispensary, Rochester, New York

In 1956 Ludwig and Goldberg[1] published an adaptation of the anthrone colorimetric technique for the quantitative analysis of carbohydrates in foods and in oral rinsings. Their method was relatively quick and accurate and an improvement over earlier carbohydrate analytical procedures, such as the Somogyi-Shaffer-Hartmann method.[2] They demonstrated that prior hydrolysis and deproteinization of the samples were not required when the anthrone technique was employed.

The major disadvantage of the method was the instability of the reagent (anthrone dissolved in concentrated sulfuric acid), which made it necessary to prepare fresh reagent daily and to restandardize it daily against a series of standard glucose solutions. Modifications in the technique, to be described in this paper, make this unnecessary and give a considerably less tedious procedure.

The use of anthrone for the analysis of carbohydrates was first suggested by Dreywood[3] in 1946. His qualitative test gave a blue-green color when anthrone reagent was added to an aqueous solution or suspension of mono-, di-, or polysaccharides. Dreywood made some investigation of the specificity of the reaction and listed eighteen carbohydrate materials, including several cellulose derivatives, which gave a positive test. He also reported a negative test with a large group of non-carbohydrates, including a variety of non-cellulose synthetic resins, organic acids, aldehydes, phenols, fats, terpenes, alkaloids, and proteins. The only non-carbohydrate material which gave a positive test was furfural, but this was not a typical positive test, in that the green color was rapidly obscured by a brown precipitate. He noted that the heat produced by the dilution of the sulfuric acid appeared to be an essential part of the test.

Morse[4] and Morris[5] adapted Dreywood's qualitative test to the quantitative analysis of carbohydrate material. The similar techniques described by these authors form the basis of all later work. It is enough to quote one of them (Morris):

> For a quantitative reaction, 2 gm. of anthrone is dissolved in 1 liter of 95 per cent sulfuric acid. . . . Four or 5 ml. of the solution to be determined is measured into a test tube of 19- to 25-mm. diameter, and 8 or 10 ml. of the reagent added. The solutions are at once thoroughly mixed by swirling. After 10 min. or more, the color is measured either in an electrophotometer against a blank containing only water and reagent or in a visual colorimeter against a glucose standard. The color varies with Beer's law, if color filters of 540mμ (green) or 620mμ (red) are used. The latter has been found preferable, since it gives higher sensitivity and decreases any error caused by extraneous colors.

This investigation was supported in part by Research Grant D-607 from the N.I.H., U.S. Public Health Service.

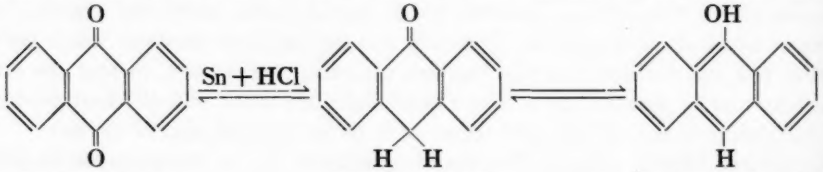
Received for publication May 27, 1960.

Morris[5] pointed out that the specificity of anthrone for carbohydrates was very high, and he reported positive reactions with all pure mono-, di-, and polysaccharides tested, as well as with all samples of dextrins, dextrans, starches, plant polysaccharides and gums, pneumococcus polysaccharides of types II and III, glucosides, and the acetates of mono-, di-, and polysaccharides. He also noted the significance of the heat of the reaction in the anthrone technique and showed that, with a given amount of carbohydrate, the intensity of the color that developed varied with the amount of heat generated.

Seifter, Dayton, Novic, and Muntwyler[6] traced discrepancies in results obtained in identical glucose samples to the rates of liberation and dissipation of heat. They largely overcame these difficulties by mixing the reagent and the test solution in tubes submerged in cold water, heating them in boiling water, and then cooling them in cold water. In their tests the curve of color development at 620 mμ reached a maximum after 9–10 minutes of heating, while more prolonged heating caused the depth of color to fall off at a considerable rate. On the basis of this finding, many subsequent workers employed a 10-minute heating period and thereby increased the accuracy of the method.

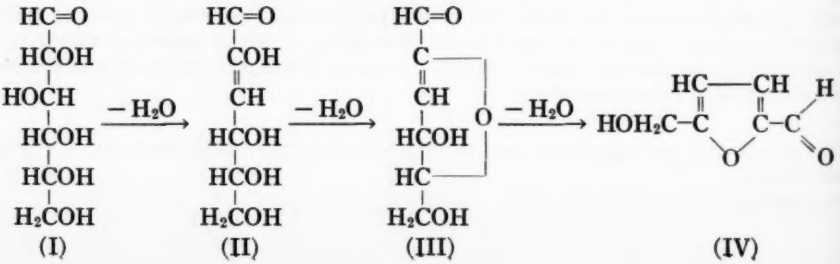
A considerable body of literature on the anthrone technique has appeared since the publication of the early studies. Specific adaptations of the test have been described for the estimation of dextran,[7] starch and cellulose,[8] methylcellulose,[9] carboxymethylcellulose,[10] carbohydrates in plant extracts,[11] streptomycin,[12, 13] deoxy sugars,[14] ribose, deoxyribose, and nucleic acids,[15] carbohydrates in the presence of serum proteins,[16] glycogen,[6] and carbohydrates in foods and oral rinsings.[1]

Chemistry of the anthrone reaction.—Anthrone, $C_6H_4COC_6H_4CH_2$, is a derivative of anthraquinone. It is produced by the catalytic reduction of anthraquinone by hydrochloric acid in the presence of metallic tin. It may exist in the keto or enol forms, known, respectively, as anthrone and anthranol. The reaction is summarized below:



Anthraquinone Anthrone (keto) Anthranol (enol)

The mechanism of the color reaction of anthrone with sugars has been studied by several investigators. Hurd and Isenhour[17] and Wolfrom, Schuetz, and Cavalieri[18] postulated that carbohydrates and carbohydrate derivatives undergo ring formation in the presence of strong mineral acids, as is shown for glucose below:



Each step in this breakdown of glucose (I) to 5-(hydroxymethyl)-2-furaldehyde (IV) represents a dehydration with either double bond or ring formation. Wolfrom *et al.* offered spectroscopic evidence for the intermediates (II) and (III) in this reaction.

Sattler and Zerban[19] suggested that the development of the green dye in the anthrone reaction depended on the presence of 5-(hydroxymethyl)-2-furaldehyde, or a similar furfural compound, formed by the action of the sulfuric acid on the carbohydrate.

Momose, Ueda, Sawada, and Sugi[20] chromotographed a benzene extract of the dye over alumina and demonstrated that the benzene-soluble part consisted of several dyes which gave different coloration with sulfuric acid. They determined the molecular weight of one of the main dyes to be approximately 530, and postulated the formula of the dye to be $C_{47}H_{30}O_3$. They therefore suggested that 3 moles of anthrone react with 1 mole of glucose as in the following:

$$3C_{14}H_{10}O + C_6H_{12}O_6 \rightarrow C_{47}H_3O_{30} + 5H_2O + CH_2O$$

From the analytical data and infrared spectrum of the dye, and when its reaction mechanism was considered, they postulated its probable structure might be 1,2,5,- or 1,3,5,-trianthronylidenepentane.

EXPERIMENTAL METHODS AND RESULTS

Adaptation of Loewus anthrone technique.—One of the major difficulties in the use of the anthrone method is the darkening of the anthrone–sulfuric acid reagent with time. To overcome this, it is necessary to prepare fresh reagent each day and make a new standardization curve or regression equation for each series of determinations. To avoid this, Loewus[21] in 1952 suggested using a solution of 2 per cent anthrone (recrystallized from benzene and light petroleum ether) in reagent grade ethyl acetate, which Loewus claimed was stable for several weeks. After 0.5 ml. of this reagent is added to 2 ml. of the carbohydrate solution, 5 ml. of concentrated sulfuric acid are carefully layered underneath, and gentle swirling used to hydrolyze the ethyl acetate, giving a floc of anthrone which redissolves as more rapid swirling mixes the contents of the tube. The advantages of the Loewus technique suggested the desirability of using it for making carbohydrate determinations in studies of food clearance from the teeth. However, in our hands, the technique as described by Loewus gave results which were unreliable and unreproducible.

Because it had been pointed out by several investigators[6–8] that the amount of green coloration produced was greatly influenced by the amount of heat of reaction of the sulfuric acid and water, the effect of controlling the temperature conditions under which the reaction took place was studied.

To check the effect of controlled heating and cooling on the accuracy of Loewus' technique, the following tests were made. Four series of glucose solutions, each containing 0 (blank, distilled water), 20, 40, 60, 80, and 100 μg/ml were reacted with the Loewus reagent and sulfuric acid (glucose solution, 2 ml.; reagent, 0.5 ml.; sulfuric acid, 5 ml.), relying solely on the heat of reaction of the acid and water to produce the green dye. These samples were allowed to stand 10 minutes following mixing and were then read in the colorimeter. Four identical series of standard glucose solutions were similarly reacted with the Loewus reagent and sulfuric acid, but in this case,

immediately following mixing, the samples were transferred to a 100° C. water bath for 10 minutes, followed by 4° C. for 5 minutes, and, to prevent condensation of moisture on the optical tubes during reading, 5 minutes in water at 20° C. All samples were read in a colorimeter* at 630 mμ against the distilled water-reagent–sulfuric acid blank, each sample being read three times, with the 100 per cent transmittance with the blank being checked between each reading.

The results (Table 1 and Fig. 1) show that the controlled heating and cooling resulted in a marked increase in accuracy and reproducibility, particularly in the higher glucose range. For instance, in the 80 μg/ml samples, the standard deviation of the

TABLE 1

EFFECT OF CONTROLLED HEATING AND COOLING OF SAMPLES ON CARBOHYDRATE DETERMINATIONS ON FOUR SERIES OF STANDARD GLUCOSE SOLUTIONS—LOEWUS MODIFICATION OF ANTHRONE TECHNIQUE

SAMPLE—STANDARD GLUCOSE (μG/ML)	PERCENTAGE TRANSMITTANCE					STANDARD DEVIATIONS
	Series 1	Series 2	Series 3	Series 4	Means	
	(A) Without Heating					
0	94.0	95.1	96.0	90.7	93.95	2.0
20	55.8	53.2	58.3	71.3	59.65	6.9
40	37.9	32.9	35.9	45.2	37.98	4.5
60	25.0	19.2	23.2	17.2	21.15	3.1
80	14.6	10.5	43.0	9.0	19.28	13.9
100	7.8	5.8	8.8	25.8	12.05	8.0
	(B) With Heating					
0	86.2	88.7	84.8	89.9	87.40	2.0
20	51.0	50.8	52.5	51.6	51.48	0.3
40	31.8	31.0	31.2	30.7	31.18	0.4
60	18.1	18.6	17.8	17.8	18.08	0.3
80	10.4	11.0	10.6	11.4	10.85	0.4
100	6.8	6.8	6.8	7.0	6.85	0.1

results of the four samples was reduced from 13.9 to 0.4. The range of percentage transmittance values obtained in this experiment is demonstrated graphically in Figure 1. The considerable increase in reproducibility obtained by controlling the temperature conditions may be visualized when the shaded areas in the two graphs are compared.

Similar tests using foodstuffs suspensions gave comparably reproducible results.

Stability of reagent.—To check on the stability of Loewus' anthrone–ethyl acetate reagent when kept in amber, glass-stoppered bottles, we made determinations of standard glucose solutions on the first day the reagent was made and then after 90 days' storage. The findings on duplicate series of glucose solutions of concentrations 0, 20, 40, 60, 80, and 100 μg/ml are given in Table 2 and Figure 2. Somewhat higher

* Bausch and Lomb.

TABLE 2

EFFECT OF AGING OF ANTHRONE REAGENT—DUPLICATE STANDARD GLUCOSE SAMPLES DETERMINED WITH REAGENT AT 90-DAY INTERVAL

SAMPLE—STANDARD GLUCOSE (μG/ML)	PERCENTAGE TRANSMITTANCE	
	Day 1	Day 90
0	{100.0 / 100.0} 100.0	{100 0 / 100.0} 100.0
20	{58.4 / 57.4} 57.9	{59.2 / 59.0} 59.1
40	{35.9 / 33.5} 34.7	{37.4 / 36.9} 37.1
60	{21.7 / 21.0} 21.4	{21.8 / 22.9} 22.4
80	{13.2 / 12.3} 12.8	{14.0 / 13.2} 13.6
100	{8.0 / 7.2} 7.6	{7.8 / 7.8} 7.8

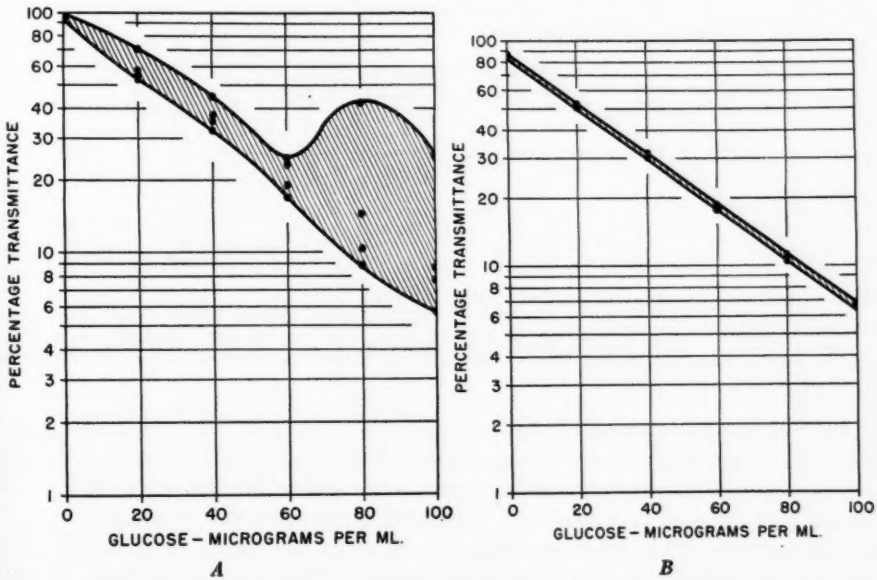


FIG. 1.—Loewus modification of anthrone technique on four series of standard glucose solutions, without and with controlled heating of the samples. In each case, the shaded area represents the extent of variations of percentage transmittance values recorded. *A*, without controlled heating; *B*, with controlled heating.

values for percentage transmitance are shown with 90-day-old reagent. However, in the light of some limitations in accuracy associated with obtaining representaive aliquots of starchy foodstuffs (discussed below), the differences with aging are unimportant enough to permit 90-day storage of the reagent.

Accuracy.—The most satisfactory method of visualizing the range of accuracy of any colorimetric system is to plot calibration data as per cent absorption* as ordinates against logarithm of concentration as abscissas—the so-called Ringbom plot. When we applied this technique to the Loewus anthrone technique with the modification of con-

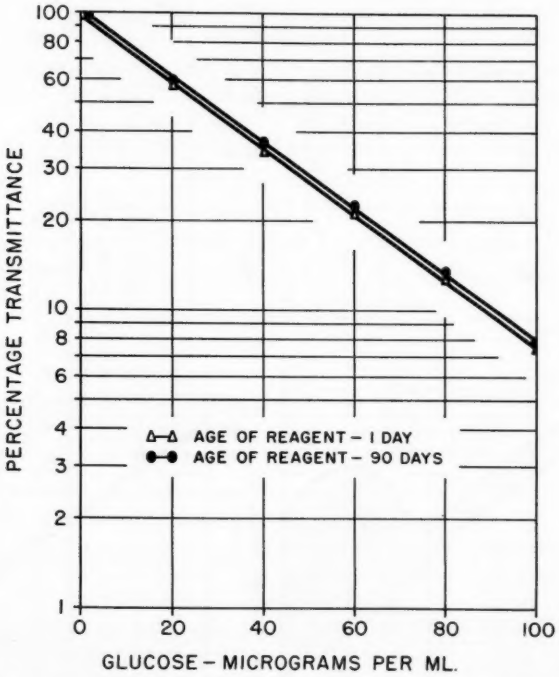


FIG. 2.—Effect of aging of anthrone reagent (anthrone dissolved in ethyl acetate) over a 90-day period on percentage transmittance values obtained with standard glucose solutions of concentrations 0, 20, 40, 60, 80, and 100 μg/ml.

trolled heating and cooling of the samples, a curve such as that shown in Figure 3 resulted. This curve is typical of any colorimetric system that conforms to Beer's law, and, as Ayres[22] points out, such a curve always passes through an inflection. The accuracy of the analytical procedure is greatest at this inflection, where the curve has its steepest slope. This inflection always occurs at the same point, represented by 63.2 per cent absorbency (36.8 per cent transmittance). This, then, is the point of maximum accuracy of the anthrone technique. At this point an error of 1 per cent in instrument reading will give an analysis error of 2.72 per cent.[22] During the dilution of the samples before they are reacted with the anthrone reagent, the concentration range should be adjusted so that it falls as closely as possible to a value which will give this

* Per cent absorbency = 100 — per cent transmittance.

per cent absorbency (approximately 38 μg/ml). Certainly, all samples should fall within the range of 40–80 per cent absorbency (60–20 per cent transmittance), which represents a concentration range of approximately 20–60 μg/ml. Beyond this range, the steepness of the Ringbom plot decreases significantly, and therefore accuracy falls off rapidly.

An investigation was made into the magnitude of the analytical errors introduced by variations in the optical characteristics (± 0.5 per cent transmittance tolerance) of the optical tubes used in this study. Details of this investigation are recorded elsewhere.[23] These errors were shown to be largely negligible, provided that the concentration of the unknown sample was adjustied to the correct range.

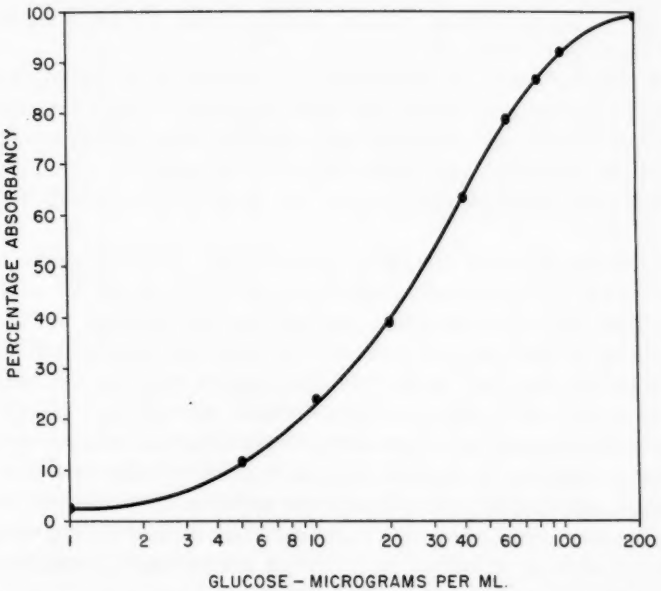


FIG. 3.—Ringbom plot. Standard curve for glucose (0, 5, 10, 20, 40, 60, 80, 100, and 200 μg/ml) plotting per cent absorbency as ordinates against logarithm of concentration as abscissas. Inflection, occurring at 63.2 per cent absorbency, represents point of maximum accuracy of the technique.

Regression equation.—To convert percentage transmittance values obtained for unknown carbohydrate solutions to carbohydrate concentrations (μg/ml), a simple calibration curve (glucose concentration as abscissa against log percentage transmittance as ordinate) obtained from data for standard glucose solutions (0–100 μg/ml) may be used. However, as was suggested by Davies[24] and has been confirmed by the present investigators, it is both more convenient and more accurate to use a regression equation,

$$Y' - (M_Y) = r \cdot \frac{\sigma_Y}{\sigma_X} \cdot (X' - [M_X])\ *,$$

* Where Y' = carbohydrate concentration of unknown (μg/ml); M_Y = mean carbohydrate concentration of standard glucose solutions; σ_Y = standard deviation of carbohydrate concentrations of standard glucose solutions; σ_X = standard deviation of optical densities (= 2 — log percentage

rather than a graph to convert the transmittance values to carbohydrate values. A full discussion of this subject has been published elsewhere.[23]

USE OF ANTHRONE TECHNIQUE FOR DETERMINATION OF CARBOHYDRATES IN FOODS

The Loewus anthrone technique with our controlled heating modification has been used extensively in this laboratory for carbohydrate analyses on foods and food/saliva mixtures and has proved quite satisfactory. However, in foodstuff work, certain additional modifications were made in the technique, notably the blending of the samples to overcome the lack of homogeneity of the suspensions (one of the important limitations in accuracy).

On the basis of our preliminary studies, outlined above, the following standard procedure was developed.

Foodstuff samples were first blended for 10 minutes in an electric blender.* For this purpose a polytron attachment† was used, which gave a high-frequency mechanical shear in addition to the usual hydraulic shearing action of the standard blender. This reduced the particulate and fibrous matter in the sample to produce a suspension that was sufficiently homogeneous to allow the obtaining of relatively equivalent aliquots.

The blended samples were diluted to a point where they fell within the range of accuracy of the anthrone reaction (approximately 20–60 μg/ml for maximum accuracy). Four 2-ml. aliquots of the diluted sample were then pipetted into 18 $\times$ 150-mm. test tubes. 0.5 ml. of the 2 per cent anthrone—ethyl acetate reagent‡ was added to each tube from a burette. Five ml. of 96.7 per cent reagent grade sulfuric acid were then carefully layered into each tube from an automatic, overflow-type pipette. The tubes were gently swirled until the ethyl acetate had hydrolyzed, as indicated by the appearance of a floc of anthrone in the tube. The contents of the tube were then thoroughly mixed by more rapid swirling to redissolve the anthrone in the sulfuric acid. As soon as all the tubes were mixed, they were transferred to a bath of boiling water, and each one was stirred with an individual glass stirring rod to insure complete mixing. The

---

transmittance) of standard glucose solutions; X' = optical density of unknown; M_X = mean of optical densities of standard glucose solutions; r = correlation coefficient

$$= \frac{M_{XY} - (M_X) \cdot (M_Y)}{(\sigma_X) \cdot (\sigma_Y)};$$

M_{XY} = mean of the products of the optical densities and carbohydrate concentrations of standard glucose solutions.

* Waring Blendor.

† Willems Polytron Attachment, Bronwill Scientific Division, Will Corporation, Rochester 10, New York.

‡ The purity of the anthrone sample being used to make the ethyl acetate reagent is important. It was found that when using one commercial sample of anthrone it was impossible to dissolve a sufficient amount of the material to produce a 2 per cent solution, presumably unless it was first recrystallized from benzene and light petroleum ether, as was suggested by Loewus (ref. 21). Other samples, however, have been of a sufficiently high degree of purity to dissolve readily in the ethyl acetate without any further preparation.

tubes were covered with glass marbles to prevent condensation of moisture in the upper part of the tubes and left in the boiling water for a total of 10 minutes. They were then transferred to an ice bath for 5 minutes and, following this, to a 20° C. bath for 5 minutes. The samples were transferred to optical tubes and read in a monochromatic colorimeter* using a 630-mμ filter. Each sample was read three times, the 100 per cent transmittance being checked and adjusted if necessary with the distilled water–reagent–sulfuric acid blank between each reading. The means of the three readings were used to derive optical density values from tables, which were substituted in the regression equation to give the carbohydrate concentrations. The mean of the four aliquots was taken as the final carbohydrate value.

Glucose equivalents per cent.—In many foodstuff studies it is necessary to know the percentage carbohydrate content of the test foods in order to convert carbohydrate values of oral rinsings, etc., to total food values. The modified anthrone procedure has been used to derive these percentage carbohydrate values of foods. However, it should be noted that the expression "percentage carbohydrate," as obtained for a foodstuff by the anthrone procedure, is something of a misnomer and would be better termed "glucose equivalents per cent." This is so because the depth of color resulting from the anthrone reaction varies with the type of sugar present. The same depth of color, for instance, is given by 100 μg. of glucose, 105 μg. of maltose, and 111 μg. of glycogen.[5] Where an unknown contains a pure sugar other than glucose, the necessary conversion factor can very simply be employed to allow for these color variations. However, where the unknown is composed of a mixture of unknown carbohydrates as in a foodstuff, such a conversion factor cannot be used, and the result is not an absolute carbohydrate percentage but a glucose equivaleint, which will vary from the true percentage carbohydrate value by some undeterminable amount. This error is of no significance when, as in our work, the glucose equivalents per cent values as previously determined in food samples are used only as a basis for converting carbohydrate values to total food values in oral rinsings, etc. For this purpose the glucose equivalents per cent is just as satisfactory an index as the absolute percentages of all the individual carbohydrates in the food.

SUMMARY

The Loewus method of carbohydrate analysis employing anthrone dissolved in ethyl acetate as the reagent was adapted for making carbohydrate determinations upon foodstuffs and food/saliva mixtures. The modified procedure was found to be satisfactory for the purpose and considerably simpler than other techniques, in that it made daily preparation and standardization of reagent unnecessary. The modification of controlled heating and cooling of the samples improved accuracy considerably. Only negligible errors were introduced by the aging of the reagent over a 90-day period. A Ringbom plot served to establish the range of maximum accuracy of the anthrone technique at 20–60 μg/ml. Methods of obtaining representative aliquots of foodstuff suspensions and of determining percentage carbohydrate values for foodstuffs were outlined.

* Bausch and Lomb.

REFERENCES

1. Ludwig, Thomas G., and Goldberg, Hyman J. V. The Anthrone Method for the Determination of Carbohydrates in Foods and in Oral Rinsings, *J. D. Res.*, **35**:90, 1956.
2. Shaffer, P. A., and Somogyi, M. Copper-iodometric Reagents for Sugar Determination, *J. Biol. Chem.*, **100**:695, 1933.
3. Dreywood, Roman. Qualitative Test for Carbohydrate Material, *Ind. and Eng. Chem., Anal. Ed.*, **18**:499, 1946.
4. Morse, E. E. Anthrone in Estimating Low Concentrations of Sucrose, *Ind. and Eng. Chem., Anal. Ed.*, **19**:1012, 1947.
5. Morris, Daniel L. Quantitative Determination of Carbohydrates with Dreywood's Anthrone Reagent, *Science*, **107**:254, 1948.
6. Seifter, Sam, Dayton, Seymour, Novic, B., and Muntwyler, Edward. The Estimation of Glycogen with the Anthrone Reagent, *Arch. Biochem.*, **25**:191, 1950.
7. Scott, Troy A., Jr., and Melvin, Eugene H. Determination of Dextran with Anthrone, *Anal. Chem.*, **25**:1656, 1953.
8. Viles, Frederick J., Jr., and Silverman, Leslie. Determination of Starch and Cellulose with Anthrone, *Anal. Chem.*, **21**:950, 1949.
9. Samsel, E. P., and DeLap, R. A. Colorimetric Determination of Methylcellulose with Anthrone, *Anal. Chem.*, **23**:1795, 1951.
10. Black, Henry C. Determination of Sodium Carboxymethylcellulose in Detergent Mixtures, *Anal. Chem.*, **23**:1792, 1951.
11. Yemm, E. W., and Willis, A. J. The Estimation of Carbohydrates in Plant Extracts by Anthrone, *Biochem. J.*, **57**:508, 1954.
12. Emery, W. B., and Walker, A. D. Chemical Assay of Streptomycin B (Mannosido-Streptomycin), *Nature*, **162**:525, 1948.
13. Kowald, J. A., and McCormack, R. B. Determination of Mannosido-Streptomycin and Dihydromannosidostreptomycin, *Anal. Chem.*, **21**:1383, 1949.
14. Koehler, Leonore H. Reaction of Deoxy Sugars with Anthrone, *Anal. Chem.*, **26**:1914, 1954.
15. Gary, N. D., and Klausmeier, R. E. Colorimetric Determination of Ribose, Deoxyribose, and Nucleic Acid with Anthrone, *Anal. Chem.*, **26**:1958, 1954.
16. Shetlar, M. R. Use of Anthrone Reaction for Determination of Carbohydrates in the Presence of Serum Protein, *Anal. Chem.*, **24**:1844, 1952.
17. Hurd, Charles D., and Isenhour, Lloyd L. Pentose Reactions. I. Furfural Formation, *J. Am. Chem. Soc.*, **54**:317, 1932.
18. Wolfrom, M. L., Schuetz, R. D., and Cavalieri, Liebe F. Chemical Interactions of Amino Compounds and Sugars. III. The Conversion of D-Glucose to 5-(Hydroxymethyl)-2-Furaldehyde, *J. Am. Chem. Soc.*, **70**:514, 1948.
19. Sattler, Louis, and Zerban, F. W. The Dreywood Anthrone Reaction as Affected by Carbohydrate Structure, *Science*, **108**:207, 1948.
20. Momose, Tsutomu, Ueda, Yo, Sawada, Kei, and Sugi, Ayako. Organic Analysis. VIII. Reaction Mechanism of Anthrone with Sugars. (1), *Pharm. Bull.* (Japan) **5**:31, 1957.
21. Loewus, Frank A. Improvement in Anthrone Method for Determination of Carbohydrates, *Anal. Chem.*, **24**:219, 1952.
22. Ayres, Gilbert H. Evaluation of Accuracy in Photometric Analysis, *Anal. Chem.*, **21**:652, 1949.
23. Beck, Donald J. *In Vitro* Acid Production and Food Retention in Relation to Dental Caries. M.S. thesis, University of Rochester, 1959.
24. Davies, G. N. Preliminary Report on Research Activities at Eastman Dental Dispensary over Period September, 1955, to May, 1956. Unpublished data, 1956.

Proteolytic Activities of Some Oral Spirochetes

*ROBERT R. OMATA and EDWARD G. HAMPP**

National Institute of Dental Research, National Institutes of Health
Bethesda, Maryland

Few studies on the metabolism of spirochetes have been published. Chang[1] reported the non-utilization of sugars and the requirement of serum for growth by *Leptospira icterohaemorrhagiae*. Marshall[2] and Helprin and Hiatt[3] studied the stimulatory effect of serum and fatty acids on the respiration of leptospirae, while Fulton and Spooner,[4] using *L. icterohaemorrhagiae*, analyzed the growth medium for chemical changes and found that accumulations of small quantities of fatty acids occurred and that there was no significant disappearance of protein during growth. Barban[5] reported transamination by the Reiter treponeme in the formation of glutamic acid, aspartic acid, alanine, and glycine. Markovetz and Larson[6] demonstrated transamination by *L. biflexa*, with pyridoxal phosphate as the coenzyme, in the formation of glutamic acid, aspartic acid, and alanine. Berger[7] reported the dissolution of the "mucin clot" from synovial fluid by culture filtrates of certain oral spirochetes, although Hampp, Mergenhagen, and Omata[8] could demonstrate no mucopolysaccharase activity by oral spirochetes. Recently the nutritional requirements of the oral treponemes and *Borrelia vincentii* were studied extensively by Hampp and Nevin,[9, 10] and a partially defined medium which contained only small amounts of ascitic fluid and several growth factors was devised.

Several preliminary experiments were performed in connection with earlier work on the enzymic activities of oral spirochetes (unpublished data) which indicated proteolytic activity of these micro-organisms. The present report concerns the utilization of protein by strains of oral treponemes and of *B. vincentii* in relation to the phases of their growth.

MATERIALS AND METHODS

The basal medium employed has been described previously[11, 12] as essentially a veal-heart infusion prepared in a sterilizer and containing thiopeptone (BBL). At the time of use, appropriate quantities of the medium were heated in a boiling-water bath to remove residual air, then enriched with 0.1 per cent reduced glutathione and canine ascitic fluid as required. The latter substance is considered to be indispensable for spirochetal growth; however, by gradual reduction of the added ascitic fluid over an extended number of transfers it was possible to adapt two strains of oral spirochetes to grow in the basal medium without it. The two primary strains used, FM, a small oral treponeme, and N9, *B. vincentii*, were maintained on the basal medium enriched

Received for publication May 31, 1960.

* Senior Research Associate, American Dental Association at the National Institute of Dental Research.

with 0.1 per cent reduced glutathione and 1 per cent canine ascitic fluid (AF medium). The two adapted variants FM-G and N9-G, were maintained on the basal medium supplemented with glutathione only (G medium).

In the experiment, multiple tubes, 15 × 125 mm., containing 14 ml. of the respective media, were each inoculated with 1 ml. of a 7-day culture of the respective strain. Uninoculated tubes were included as controls. All tubes were closed with sterile rubber stoppers to exclude air and incubated at 35° C.

At intervals of 2–21 days, duplicate tubes of each culture and of its uninoculated controls were removed for analysis. The number of spirochetes was determined by direct counting of suitable dilutions in a Petroff-Hauser chamber. Each count was the average of at least four determinations.

Five ml. from each culture tube and control tube were centrifuged at 5° C. to remove spirochetes and suspended protein of the medium. Half of each supernatant was treated with an equal volume of 10 per cent trichloracetic acid (TCA) and centrifuged at 5° C. to remove precipitated proteins. "Protein values" were determined on all supernatants by the method of Lowry, Rosebrough, Farr, and Randall.[13] The true protein content of each tube was considered to be the difference between the protein value of the untreated supernatant and the protein value of the corresponding TCA-treated supernatant. These results were substantiated in pilot experiments by direct determination of the protein values of the TCA-precipitated protein. The average protein contents of the uninoculated controls were 3.1 mg/ml for the G medium and 3.7 mg/ml for the AF medium.

In addition, amino acid values were determined on the TCA-treated supernatants by the procedure of Rosen.[14] The results on the uninoculated controls (G and AF media) were strikingly similar, ranging from 5.9 to 6.5 mg. of leucine equivalents per milliliter.

RESULTS

The data presented in Table 1 are the results of the proteolytic activity of the oral spirochetes correlated with cell counts at various time intervals. Since the degree of proteolytic activity of the various strains of micro-organisms up to the seventh day was related to the number of cells present at the time of analysis of the culture supernatants and since some variations were observed at later periods, it is expedient to discuss the characteristics of their growth phases. At the time of inoculation of the cultures, the initial cell counts for *B. vincentii* N9 and its variant N9-G were 13.3×10^6, and the small oral treponeme FM and its variant FM-G were 24×10^6 cells per milliliter.

The growth phases of both strains of *B. vincentii* in their respective media did not differ greatly from the second through the fourteenth days. The logarithmic phases in both instances occurred between the second and seventh days, and the points of stationary phase were reached by the fourteenth day. By the twenty-first day, it was difficult to count the cells in the G medium because of the formation of spirochetal granules and some loss of cellular integrity, resulting in lower total cell counts. On the contrary, however, successful enumeration of cells in the AF medium was possible because the organisms retained their morphologic characteristics, with only limited granule formation, and showed a slight increase in the total number of cells, as evidenced in the table.

The proteolytic activity of the two strains of *B. vincentii* (N9) and its variant (N9-G) was not manifested during the second day of growth; on the contrary, there were slight increases in the protein values in both the AF and the G media. An explanation for the observation is not available and is seemingly not consistent with an error in protein analysis. However, by the fourth day, medium AF showed a decrease in protein, whereas the G medium still retained an increased protein value, although it was roughly half that for the preceding time interval. During this period, however, there were increased amino acid values, as indicated in Table 1. When the peak of the logarithmic phase was attained on the seventh day by either strain of spirochetes in

TABLE 1*

CELL COUNTS AND PROTEOLYTIC ACTIVITIES OF ORAL SPIROCHETES

DAYS	AV. CELL COUNT $N \times 10^6$/ML	PER CENT CHANGE IN PROTEIN	PER CENT CHANGE IN AMINO ACIDS	AV. CELL COUNT $N \times 10^6$/ML	PER CENT CHANGE IN PROTEIN	PER CENT CHANGE IN AMINO ACIDS
	Borrelia vincentii Strain N9 (AF Medium)			*Borrelia vincentii* Strain N9-G (G Medium)		
0	13.3	.....	.....	13.3	.....	.....
2	67.5	+11.9	+ 6.7	67.5	+ 17	+ 9.5
4	177.5	− 2.9	+ 3.1	170	+ 9	+ 11.1
7	220	−25.7	+ 17.5	237.5	− 47.5	+ 22.4
14	287	−12.5	+ 28.9	283.5	− 18.3	+ 54.5
21	313	− 6.4	+ 58.1	180	+112.8	+ 59.5
	Small Oral Treponeme Strain FM (AF Medium)			Small Oral Treponeme Strain FM-G (G Medium)		
0	24	.....	.....	24	.....	.....
2	270	−45.2	+ 20	262.5	− 19	+ 26.2
4	530	−48.1	+ 57.7	502.5	− 44.4	+ 45.6
7	700	−95.1	+ 95.1	721	− 65.2	+ 93.6
14	731.5	−66.2	+ 96.9	697.5	− 63.8	+ 82.3
21	714	−46.4	+111.6	581	− 52.5	+140.5

* + = percentage increase in protein and amino acid contents of the culture media; − = percentage decrease in protein contents of the culture media.

their respective media, the greatest percentage decrease in protein content occurred. Concomitantly, there were increased amounts of amino acids in both instances. By the fourteenth day, a declining proteolytic activity was evident, although the amino acid values continued to increase. On the twenty-first day, the AF culture showed a slight decrease in proteolysis and a twofold increase in the amino acid values compared with the preceding period; the former observation may possibly be due to the organisms reaching the stationary growth phase. However, the G culture medium showed only a slight increase in the amino acids, but the protein content was markedly increased. This change may possibly be associated with the low cell count, excessive granule formation, and loss of cellular integrity with autolysis, as previously indicated for this period of growth.

The small oral treponemes (FM and FM-G) in their respective media generally followed the growth phases of the *Borrelia* strains; however, the total cell counts were

much greater at any given time period (Table 1). The logarithmic phases of growth in AF and G media occurred between the second and seventh days, and the total cell counts in both instances were comparable. The points of stationary phase were reached during the seventh and fourteenth days. Declining cell counts were observed in the G medium by the fourteenth day, and at the twenty-first day marked changes occurred in cellular morphology associated with excessive granule formation, as was also observed with *B. vincentii* (N9-G).

The small oral treponemes showed greater proteolytic activity in their respective media at the selected time intervals than did the strains of *B. vincentii*. Proteolysis was evident as early as the second day in both the AF and the G media and was greater in the former; however, the amino acid values were comparable. The proteolysis in both media progressed at an increased rate during the logarithmic phase of growth and reached the peak of activity on the seventh day, which is also indicated by the increased amounts of amino acids in both culture supernates. From the seventh to the fourteenth days, the amino acid values remained constant in both cultures; however, the AF broth showed decreased proteolytic activity, and the G medium remained essentially unchanged. At the twenty-first day, both AF and G cultures showed declining proteolysis; however, the amino acid values were markedly increased in the G medium and to a lesser degree in the AF medium.

DISCUSSION

Ascitic fluid or serum supplements have been shown to be essential components for the isolation and cultivation of the family Treponemataceae.[12, 15–21] However, preliminary experiments indicated that the usual 10 per cent amounts of ascitic fluid employed for culturing these organisms complicated the protein and amino acid analyses. Therefore, eventually both parent strains of spirochetes (FM and N9) were successfully grown in the basic medium with reduced amounts of ascitic fluid (1 per cent). After numerous transfers, these organisms were eventually adapted to grow without ascitic fluid (FM-G and N9-G). The cell crops for all strains were comparable to those obtained with 10 per cent ascitic fluid in the basic medium. The mechanism of the adaptive process in the present study is not clear. Hampp and Nevin[9] showed that the parent strains could be grown in the heat-altered basal medium, provided that additional growth factors were added to substitute for heat-labile constituents of the medium and ascitic fluid.

The demonstration of proteolytic activity by oral spirochetes supplies additional information on the metabolic activity of this group of micro-organisms. The differences in the proteolysis of the *Borrelia* strains and the strains of oral treponemes indicate variations in their nitrogen metabolism. Strains N9 and N9-G appear capable of using nitrogen-containing substrates smaller than the TCA-precipitable proteins—that is, TCA-soluble compounds, possibly peptides and polypeptides, which are provided in the culture media by thiopeptone and the veal-heart infusion. The small net change in the protein values and the smaller percentage of liberation of amino acids by the *Borrelia* strains suggest the elaboration of peptidases by these organisms. The small oral treponemes, FM and FM-G, showed greater proteolytic activity than did N-9 and N9-G strains by degrading greater amounts of TCA-precipitable components or the larger protein molecules, which are mainly provided in the media by the veal-heart in-

fusion and ascitic fluid. The "proteinase" activity of the treponemes was indicated by the greater diminution of proteins and the greater over-all increase in the liberation of amino acids over the growth range.

Diminished proteolytic activity of all strains of organisms commenced during the stationary phase of growth and was more manifest in the G medium. At the 21-day interval, the formation of spirochetal granules, the loss of cellular integrity, and the lower cell counts may in part be due to the diminished amounts of oleic and other fatty acids in the G medium in contrast to AF medium. Evidence was shown by Nevin and Hampp[10] that in the absence of added oleic acid in their partially defined medium there were greater numbers of granules formed by these spirochetes.

SUMMARY

The proteolytic activities during the growth phase of two strains of *B. vincentii* and two of a small oral treponeme were demonstrated. The parent strain of *B. vincentii* (N9) and the adapted variant (N9-G), which was adapted to grow in the absence of ascitic fluid, grew equally well in their respective media and showed evidence of significant proteolytic activity. Cultures of the two strains of the small oral treponeme (FM and FM-G) showed greater proteolytic activity than did the *B. vincentii* strains.

The greatest degree of proteolysis by all strains was observed to coincide with the logarithmic growth phases, occurring during the second through the seventh days. During the stationary phases, there were diminished activities, especially with the *Borrelia* strains.

REFERENCES

1. Chang, S. L. Studies on *Leptospira icterohaemorrhagiae*. III. The Growth Rate of and Some Biochemical Observations on *L. icterohaemorrhagiae* in Culture, *J. Infectious Diseases*, **81**:35, 1947.
2. Marshall, P. B. Measurement of Aerobic Respiration in *Leptospira icterohaemorrhagiae*, *J. Infectious Diseases*, **84**: 150, 1949.
3. Helprin, J. J., and Hiatt, C. W. Effect of Fatty Acids on the Respiration of *Leptospira icterohaemorrhagiae*, *J. Infectious Diseases*, **100**:136, 1957.
4. Fulton, J. D., and Spooner, D. F. Metabolism of *Leptospira icterohaemorrhagiae* in Vitro, *Exptl. Parasitol.*, **5**:154, 1956.
5. Barban, S. Studies on the Metabolism of the *Treponemata*. II. Transamination in the Reiter Treponeme, *J. Bacteriol.*, **71**:274, 1956.
6. Markovetz, A. J., and Larson, A. D. Transamination in *Leptospira biflexa*, *Proc. Soc. Exptl. Biol. Med.*, **101**:538, 1959.
7. Berger, U. Über Hyaluronidasebildung durch "saprophytische" Treponemen, *Zentr. Bakteriol., I. Orig.*, **165**:563, 1956.
8. Hampp, E. G., Mergenhagen, S. E., and Omata, R. R. Studies on Mucopolysaccharase Activity of Oral Spirochetes, *J. D. Res.*, **38**:979, 1959.
9. Hampp, E. G., and Nevin, T. A. Substitution of Known Compounds for Ascitic Fluid in the Cultivation of *Borrelia vincentii*, *J. Bacteriol.*, **77**:800, 1959.
10. Nevin, T. A., and Hampp, E. G. Partially Defined Medium for the Cultivation of *Borrelia vincentii*, *J. Bacteriol.*, **78**:263, 1959.
11. Hampp, E. G., Scott, D. B., and Wyckoff, R. W. G. Morphologic Characteristics of Certain Cultured Strains of Oral Spirochetes and *Treponema pallidum* as Revealed by the Electron Microscope, *J. Bacteriol.*, **56**:755, 1948.
12. Fitzgerald, R. J., and Hampp, E. G. Inhibition of Oral Spirochetes by Antibiotic Agents *in Vitro*, *J. D. Res.*, **31**:20, 1952.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem.*, **193**:265, 1951.
14. Rosen, H. A Modified Ninhydrin Colorimetric Analysis for Amino Acids, *Arch. Biochem. Biophys.*, **67**:10, 1957.

15. NOGUCHI, H. Cultural Studies on Mouth Spirochetes (*Treponema microdentium* and *macrodentium*), *J. Exptl. Med.*, **15**:81, 1912.
16. KAST, C. C., and KOLMER, J. A. Methods for the Isolation and Cultivation of Treponemes with Special Reference to Culture Media, *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **24**:671, 1940.
17. ROSEBURY, T., and FOLEY, G. Cultivation of Spirochetes of the Mouth, *J. D. Res.*, **21**:308, 1942.
18. WICHELHAUSEN, D. W., and WICHELHAUSEN, R. H. Cultivation and Isolation of Mouth Spirochetes, *J. D. Res.*, **21**: 543, 1942.
19. HAMPP, E. G. A Method for Routine Isolation and Cultivation of the Smaller Oral Treponemes, *J.A.D.A.*, **30**:1066, 1943.
20. ———. *Borrelia vincentii:* Isolation, Pure Cultivation, and Morphologic Characteristics, *J. D. Res.*, **36**:660, 1954.
21. LITTLE, P. A., and SUBBAROW, Y. Use of Refined Serum Albumin as a Nutrient for *T. pallidum*, *J. Immunol.*, **50**:213, 1945.

Penetration of Carcinogenic Hydrocarbons into Oral Tissues as Observed by Fluorescence Microscopy

JOHN J. SALLEY

Department of Oral Pathology, Medical College of Virginia School of Dentistry, Richmond, Virginia

The successful production of squamous-cell carcinoma of the oral tissues has been reported previously from this and other laboratories.[1–4] The method by which carcinogenic compounds gain access to oral epithelial cells subsequent to topical application has been a subject of interesting speculation. Levy[2, 5] and Kolas[6] have cited the possible need for epithelial adnexa to serve as portals of entry. Goldhaber[7] has mentioned the presence of pre-existing mucosal ulceration as a means of entry of chemical carcinogens into oral mucosa.

Inasmuch as the carcinogenic hydrocarbons exhibit a characteristic fluorescence when exposed to ultraviolet irradiation, fluorescence microscopy has been utilized as a method of determining the distribution of these compounds in tissues. Simpson and Cramer[8] in this country and Doniach, Mottram, and Weigert[9, 10] in England have studied cutaneous tissue of mice with this technique and reported high concentrations of carcinogens in sebaceous glands shortly after topical application. From these reports a concept of the role of skin accessory structures as requisites to experimental epidermal tumor formation has arisen.[11] A discussion of this facet of cutaneous epithelial carcinogenesis and its possible relationship to oral epithelium will be presented elsewhere.[12]

The present investigation was undertaken to study the distribution of two carcinogenic hydrocarbons topically administered to epithelia with and without adnexal structures.

EXPERIMENTAL METHODS

A total of 130 Pee Dee strain* albino hamsters was divided into two groups. The first group of 50 animals was treated triweekly for 15 weeks with a 0.5 per cent solution of 9,10-dimethyl-1,2-benzanthracene in a mineral-oil solvent. The following anatomical sites were exposed by techniques previously described:[1, 4] cheek pouch mucosa, palate, tongue, and skin of the back. A second group of 80 hamsters received 3,4-benzpyrene in mineral oil for 25 weeks. Successive sacrifice was performed in each group 24 hours subsequent to each painting. Two animals of each group were sacrificed by chloroform inhalation after the initial second, third, and fourth applications and one each after the remaining paintings.

This study was supported by grant No. 308 from the Damon Runyon Memorial Fund for Cancer Research.

Received for publication May 31, 1960.

* Obtained from Pee Dee Farms, Driver, Virginia.

Immediately after sacrifice, thin oval sections of pouch, stripped palatal mucosa, a cross-section of tongue, and a 0.5-sq. cm. section of treated skin were dissected and fixed in 10 per cent neutral formalin for 3 hours. After washing with water, the tissues were infiltrated and imbedded in gelatin, U.S.P. Gelatin blocks were hardened in 10 per cent neutral formalin for 6 hours and mounted on a freezing microtome.* Sections of each specimen were cut at 10 μ and mounted in glycerogel on quartz slides.

Fluorescence microscopic observations were made in a darkened room using a uniocular microscope† equipped with apochromatic objectives, a quartz condenser, and a front surface mirror. A UV protection filter was inserted into the ocular, and the ultraviolet light source was a mercury-vapor bulb‡ mounted in an optical illuminator§ fitted with quartz optics. Light filtration was by means of a heat-absorbing filter|| and filter No. 5840,|| the latter giving maximum UV transmission at a range of 320–380 mμ Photomicrographs were exposed using 35-mm. daylight film# with an average exposure time of 6 minutes. Black-and-white illustrations presented in the following section were printed from these color transparencies. Photomicrographs for orientation[13] and to rule out the presence of ulceration were taken in the same manner but without light filtration, so that the available light in the visible spectrum served as principal light source.

In order to rule out possible artifacts based on autofluorescence due to formalin fixation,[14] sections of the tissues under study from untreated animals were prepared and observed in a similar fashion.

RESULTS

Results of this investigation are best presented in the accompanying photomicrographs as well as by the anatomical site treated. Essentially the same observations relative to carcinogen distribution were made in both groups of hamsters, so that no further distinction between the compounds used is necessary.

Skin.—The findings observed in skin removed from the intrascapular area of the back were in agreement with those reported by Simpson and Cramer[8] and Doniach *et al.*[9, 10] Carcinogens detectable by their fluorescence were observed early (one treatment) in sebaceous glands and were noted in heavy concentration in the epidermis after two to three applications (Fig. 1).

Pouch.—Figure 2 illustrates a normal, untreated hamster pouch where autofluorescence is noted within the mucous membrane. The hydrocarbon solution became incorporated in the keratin layer after a single application (Fig. 3), where it fluoresced with a brilliant yellow color. A pouch examined after three applications is seen in Figure 4 and illustrates the presence of two large fluorescent bodies in the lamina propria beneath an intact epithelial surface. A specimen removed after eight applications is shown in Figure 5. Here a large droplet of solution is noted on the surface above another fluorescing body in the lamina propria. There was a change in color to yellow-blue fluorescence when the material was noted inside the tissue.

* E. P. Leitz Co., New York, N.Y.

† American Optical.

‡ General Electric AH-4.

§ American Optical Illuminator No. 370A.

|| Owens-Corning Corp., Corning, N.Y.

Ektachrome Daylight film.

The latter observation may denote some chemical change in the hydrocarbon as postulated by Simpson and Cramer.[8]

Palate.—Penetration of palatal epithelium occurred after four treatments with either 9,10-dimethyl-1,2-benzanthracene or 3,4-benzpyrene and was detected by fluorescence of cells in the lamina propria. Figure 6 shows a section of untreated palatal epithelium, while Figure 7 illustrates a nucleated cell in the lamina propria with bluish-yellow fluorescence of the cytoplasm, with no detectable fluorescence of

FIG. 1.—Photomicrograph of section of skin after two topical applications of 3,4-benzpyrene. Note brilliant fluorescence of carcinogen on surface and to a lesser extent in the sebaceous glands below. (Mag. ×100.)

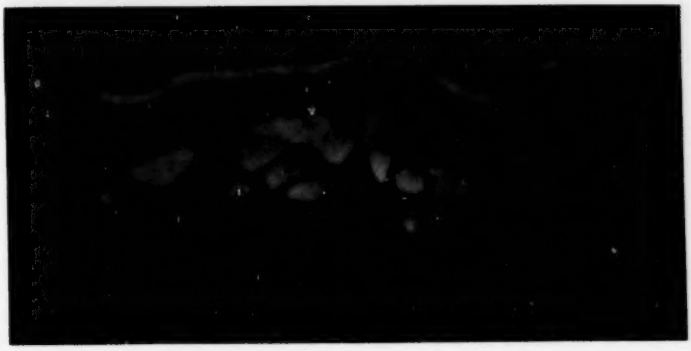


FIG. 2.—Normal, untreated hamster pouch as seen by fluorescence microscopy. (Mag. ×100.)

FIG. 3.—Section of hamster pouch 24 hours after one application of 9,10-dimethyl-1,2-benzanthracene. A deposit of the carcinogen is present on the surface in a fold. This specimen exhibits a brilliant yellow fluorescence. (Mag. ×220.)

FIG. 4.—Unbroken epithelium lining a pouch after three paintings of 3,4-benzpyrene. Three fluorescing bodies are seen in the lamina propria which have a bluish-yellow color. (Mag. ×100.)

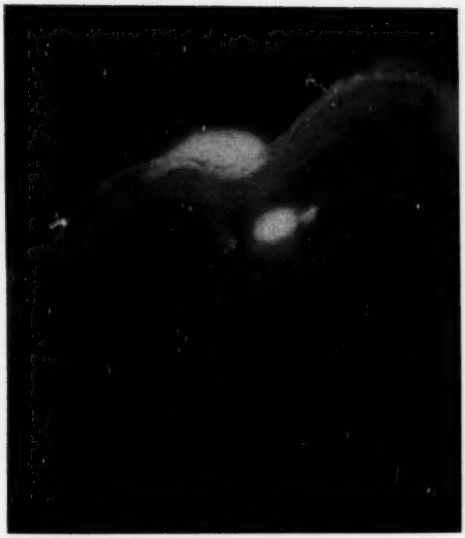


FIG. 5.—Pouch wall after eight applications of hydrocarbon solution. Fluorescence is noted both on the surface and in the lamini propria. (Mag. ×100.)

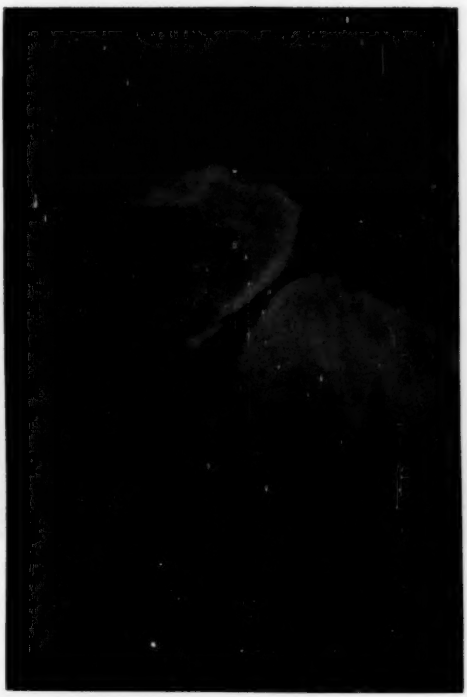


FIG. 6.—Normal, untreated palatal mucosa as observed by fluorescence microscopy. (Mag. ×100.)

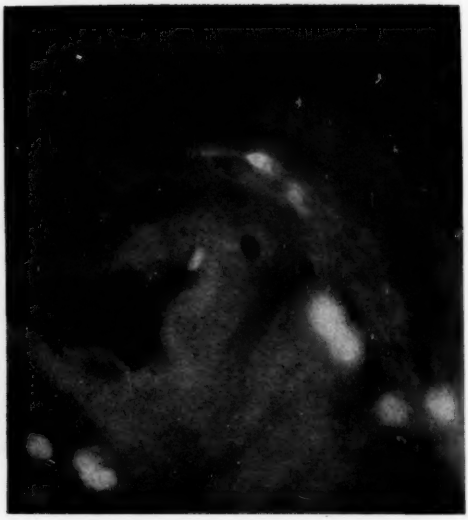


FIG. 7.—After three applications of the carcinogen, fluorescence is noted in the lamina propria of the palatal mucosa. A bluish-yellow fluorescing cell with no fluorescent material in its nucleus is seen. (Mag. ×100.)

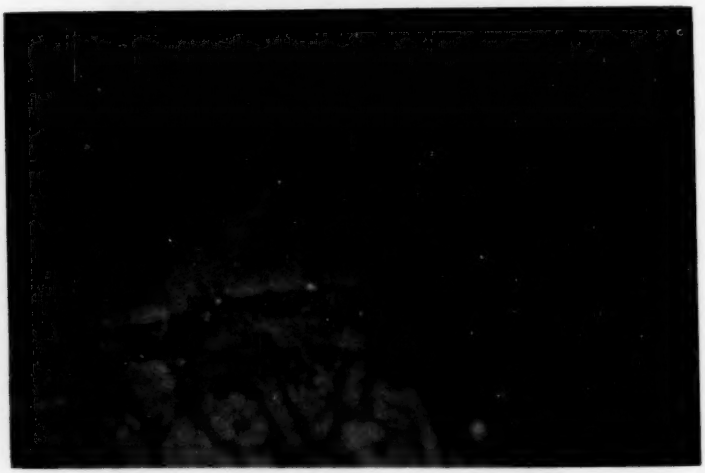


FIG. 8.—Fluorescence micrograph of the tongue after four applications of benzpyrene. Several small fluorescing particles may be seen along the basement membrane and in the lamina propria. (Mag. ×100.)

the nucleus. As was the case in the pouch, the continuity of the overlying palatal epithelium was not broken.

Tongue.—Lingual epithelium also demonstrated fluorescent particles along the basement membrane and in the lamina propria (Fig. 8). This finding was noted after three to four applications, and again a color change from yellow to blue-yellow fluorescence was noted when the detectable carcinogen was seen inside the tissue.

Specimens removed from hamsters after more prolonged treatment were essentially similar to those described above. In general, then, it would appear that carcinogens detectable by their fluorescence were noted in oral tissues after three to four paintings.

DISCUSSION

Aside from the demonstration that carcinogenic hydrocarbons can apparently be absorbed through intact mucous membrane when repeated topical applications are made, it is interesting to note that, with the exception of skin, little or none of the fluorescent material was observed intra-epithelially in this study. One may ponder the question relating to action of the carcinogen on epithelial cells proper. It may be that absorption of the chemical into the cell or cells may take place as the compound is passing through the epithelium and that the amount necessary for alteration of the cell toward malignancy is undetectable by the method utilized. The color change in fluorescence between that seen outside the tissue and that seen inside may give some support to this suggestion.

Many of the fluorescent bodies observed in the lamina propria appeared to be nucleated, like the one illustrated in Figure 7. Because of the necessity for using unstained preparations and the thickness of frozen sections, it was not possible to characterize these cells, although some appeared to be similar in size to large histocytes. This is an area which warrants additional study in light of the absence of connective tissue neoplasms when hydrocarbon compounds are topically applied.

These results would also question the absolute need for a physiologic and/or pathologic portal of entry into oral epithelium. It would seem that the response of a given type of tissue to carcinogenic action is governed by other factors. This, too, is a phase of oral cancer research which needs further exploration.

SUMMARY

One hundred and thirty albino hamsters were successively sacrificed 24 hours after each topical application of either 9,10-dimethyl-1,2-benzanthracene or 3,4-benzpyrene. Examination of skin, pouch, tongue, and palate was performed using fluorescence microscopy to trace the distribution of these carcinogens. It was noted that detectable fluorescence of the carcinogenic material was present in skin after one painting and in the oral mucous membranes following three to four applications.

REFERENCES

1. Salley, J. J. Experimental Carcinogenesis in the Cheek Pouch of the Syrian Hamster, *J. D. Res.*, **33**:253, 1954.
2. Levy, B. M. The Experimental Production of Carcinoma of the Tongue in Mice, *J. D. Res.*, **37**:950, 1958.
3. Morris, A. L. Experimental Premalignant and Early Malignant Oral Epithelium in the Syrian

Hamster, *Proc. 25th Year Celebration, University of Rochester Dental Research Fellowship Program*, p. 81. Rochester, N.Y.: University of Rochester Press, 1957.
4. SALLEY, J. J., and KRESHOVER, S. J. The Effect of Topical Application of Hydrocarbons to the Palatal Mucosa of the Hamster, *Oral Surg., Oral Med., Oral Path.*, **12**:501, 1959.
5. LEVY, B. M. The Experimental Production of Oral Malignancies, *J.A.D.A.*, **54**:514, 1957.
6. KOLAS, S. Investigation of Normal Human Saliva for Possible Anticarcinogenic Action and Chemical Carcinogenesis in Mucous Membranes, *Oral Surg., Oral Med., Oral Path.*, **8**:1192, 1955.
7. GOLDHABER, P. Further Studies on Experimental Oral Carcinogenesis, *J. D. Res.*, **37**:18, 1958.
8. SIMPSON, W. L., and CRAMER, W. Fluorescence Studies of Carcinogens in Skin. I. Histological Localization in Mouse Skin after a Single Application, *Cancer Research*, **3**:362, 1943.
9. DONIACH, I., MITTRAM, J. C., and WEIGERT, F. The Fluorescence of 3,4 Benzpyrene *in Vivo*. I. The Distribution of Flourescence at Various Sites, Especially the Skin of Mice, *Brit. J. Exptl. Pathol.*, **24**:1, 1943.
10. ———. The Fluorescence of 3,4 Benzpyrene *in Vivo*. II. The Interrelationship of the Derivatives Formed at Various Sites, *ibid.*, p. 9.
11. COWDRY, E. V. *Cancer Cells*. Philadelphia: W. B. Saunders Co., 1955.
12. SALLEY, J. J. Epithelial Carcinogenesis in the Absence of Accessory Structures, *Oral Surg., Oral Med., Oral Path.*, in press.
13. HOUSTON, M. Photographic Technics for Fluorescent and White Light Observations of the Same Tissue Section, *J. D. Res.*, **37**:20, 1958.
14. JONES, R. MCCLUNG. *Microscopical Technique*. 3d ed.; New York: Paul B. Hoeber, Inc., 1956.

Enamel Decalcification in Food-Saliva Mixtures

NARENDAR N. SONI and BASIL G. BIBBY
Eastman Dental Dispensary, Rochester, New York

A variety of attempts has been made to establish differences between the cariogenicity of different foods. These have compared sugar concentration in the saliva,[1] food retention on the teeth,[2, 3] acid formation in plaques,[4] acid production from foods in saliva,[4, 5] combinations of these,[6] and other methods. None of the procedures has produced definitive results which could serve as the basis for practical programs of caries prevention. Recent findings indicating differences in enamel-decalcifying effects of various organic acids[7, 8] or types of breads[9] and sugar[10] and the protective effects of phosphates against animal caries[11–13] suggest that more significant comparisons between foods could be obtained by making direct measurements of enamel decalcification in incubated food and saliva mixtures.

EXPERIMENTAL METHODS

Because polarization microscopy can provide information on the extent of decalcification of the hard tissues, it was decided to employ it to compare the extent of enamel decalcification produced in tooth sections incubated in food-saliva mixtures.

Plano-parallel polished sections 75–100μ thick were made from intact young human permanent teeth extracted from Rochester patients under the age of twenty years. One side of the finished sections was glued by Duco cement to a microslide. Thereafter a small area of enamel about 2 mm. in diameter was covered with moistened Burlow dry foil, and the rest of the section was covered by a thin layer of Duco cement. After 30 minutes, when the cement was fully set, the foil was removed, thus providing an open area, or window of enamel, which alone was unprotected by the cement. These assemblies were then washed in running water for half an hour. To check acceptability of the "windows" and provide a base line for comparisons, they were examined under polarized light, using water as an imbibing media. Thereafter they were placed in a plastic jar containing one of the food-saliva mixtures to be tested. The test mixtures were made by mixing 2.5 gm. of food, or 2.5 ml. in case of liquid food, with 5 ml. of whole paraffin-stimulated saliva in a commercial type "Wig-L-Bug" for 5 minutes. Natural sugars, raspberry jam, and Kraft caramel were diluted to a 10 per cent sugar level and then mixed in 5 ml. of saliva. The saliva used was collected under paraffin stimulation from one individual at 10 A.M. of each day on which tests were run.

After recording the initial pH with a Beckman zeromatic pH meter, the plastic jars were incubated at 37° C. in a water bath subjected to constant gentle rocking. The sections were removed at 6, 8, and 24 hours, washed, and examined under polarized light with the prisms at 45° for evidence of decalcification, which was recognized by

This investigation was supported in part by the Colgate-Palmolive Company.

Received for publication June 2, 1960.

changes in birefringence in the exposed "window" area, which would contrast distinctly with the adjacent protected enamel. The first signs of change appeared in the dentinal portion of the enamel. The extent of decalcification was recorded as follows: 0 = no change; 0? = questionable change; + = involving dentinal third of enamel; ++ = involving up to middle of enamel; +++ = involving up to superficial enamel.

Each time the sections were examined, pH determinations of the food-saliva mixture were also made. At the conclusion of each experiment, terminal titratable acidity was determined in the total food-saliva mixture by bringing the pH to 7.0, with 0.1 N NaOH solution.

TABLE 1

ACID FORMATION AND ENAMEL DECALCIFICATION BY FLOURS, FLOUR PRODUCTS, AND CEREALS

PRODUCT	PH/DECALCIFICATION				ML. ACID FORMED
	0 Hours	6 Hours	8 Hours	24 Hours	
Wheat flour	6.6/−	4.5/?+	4.4/++	4.5/+++	2.52
Wheat germ	6.7*/−	5.1/−	5.0/−	4.8/−	10.30
Wheat flour + 2 per cent dicalcium phosphate	6.5/−	4.5/−	4.5/−	4.4/−	4.15
Wheat flour + 2 per cent wheat-germ oil	6.0/−	4.4/−	4.4/−	4.4/−	3.05
Rye flour	6.7/−	4.8/−	4.5/−	4.6/−	3.82
Corn flour	6.9/−	5.0/−	4.8/−	4.8/?+	1.18†
Mandioca secca (granular)	5.9/−	5.2/−	5.1/−	5.1/−	1.62
White bread (regular)	6.5/−	4.8/−	4.7/+	4.7/+	1.97
White bread (special)	6.6/−	4.7/−	4.5/+	4.5/++	2.1†
Whole-wheat bread	6.4/−	4.9/−	4.8/−	4.5/+	1.89
Oil-sprayed cracker	7.3*/−	4.7*/+	4.6*/++	4.4/++	1.5†
Cracker (salted)	7.1/−	5.3/−	5.1/−	4.9*/++	1.31
Shortbread cookie	7.8/−	4.8/−	4.6*/−	4.6*/−	1.73†
Fig cookie	6.9/−	5.2/−	4.6*/+	4.9*/++	1.36†
Cake (golden loaf)	7.5/−	4.9/−	4.7/−	4.6/−	2.85
Corn flakes	6.5*/−	6.4/−	6.3/−	5.7/−	0.76†
Puffed rice	6.8/−	4.6/−	4.6/+	4.7/++	1.71†
Shredded wheat	6.9/−	4.7*/−	4.6/−	4.7/?+	2.85†

* pH readings differed by more than 0.2.

† Individual titrations differed by more than 10 per cent.

RESULTS

The averaged results obtained in two, or sometimes three, individual runs for each food are presented in Tables 1 and 2. In most instances the duplicate tests gave similar results, but where significant differences occurred, they are indicated in the tables. pH's are given to the nearest lower tenth unit, and decalcification scores, which never differed by more than one unit, to the next higher level.

From the tables it is quite apparent that foods have markedly different decalcifying effects on enamel and that there is no consistent relationship between the amount of acid formed and the amount of decalcification produced in the enamel section. Of the cereal foods tested (Table 1), wheat flour produced most decalcification, followed by a special white bread, puffed rice, two types of crackers, and one or two sorts of sweet

cookies. In this respect, the differences between wheat flour and wheat germ and flour with added phosphate are outstanding. Contrasting results are also seen between the cake and the fig or shortbread cookies and the shredded wheat and the puffed wheat cereals.

Several of the sugar products (honey, a carbonated beverage, orange juice, and jam) listed in Table 2 gave the strongest decalcification, but this may be related to the original acidity of the foods themselves. Lack of relationship between acid production and decalcification is obvious when the figures for sugar are compared with those for caramel, the chocolates, the two milks, ice cream, and chocolate pudding. It is noteworthy that, in general, more acid was produced from the flour products than from the sugar-containing foods.

In Figure 1 the final pH of the fermented food mixtures is compared with the degree of observed decalcification. It is apparent that there is no direct relationship.

TABLE 2

ACID FORMATION AND ENAMEL DECALCIFICATION BY SUGAR AND MILK PRODUCTS

PRODUCT	PH/DECALCIFICATION				ML. ACID FORMED
	0 Hours	6 Hours	8 Hours	24 Hours	
10 per cent sucrose	7.1/−	5.0/−	4.8/−	4.4/+	0.27†
Honey (10 per cent sugar)	6.9/−	4.7/−	4.5/+	4.5/+++	0.76†
Maple syrup	7.3/−	4.9/−	4.8/−	4.8/−	0.58
Cola beverage	6.6*/−	5.0/−	4.7*/−	4.6/++	0.49
Citrate beverage	5.4/−	4.9/?+	4.7/+	4.4/+++	0.48
Orange juice	5.4/−	4.8/+	4.7*/++	4.4*/+++	1.1
Raspberry jam	5.5/−	4.6/+	4.5/++	4.5/+++	0.91
Caramel (10 per cent sugar)	6.8/−	6.0/−	5.2/−	4.8/+	1.27
Dark chocolate	6.2/−	5.2/−	4.9*/−	4.8/−	2.63
Milk chocolate	6.6/−	5.1/−	4.9/−	4.7/−	3.08
Chocolate pudding	7.0/−	4.9/−	4.7/−	4.4/−	3.74
Ice cream	6.9/−	5.3*/−	4.6/−	4.7/+	2.93
Chocolate milk	7.0/−	4.5/−	4.5/−	4.5/−	2.64
Homogenized milk	7.0/−	4.9/−	5.0/−	5.0/−	1.59

* pH readings differed by more than 0.2.

† Individual titrations differed by more than 10 per cent.

DISCUSSION

A number of the findings deserve comment or emphasis. The most important of these is the lack of relationship between the amount of acid formed in food-saliva fermentation mixtures and the extent of the decalcification produced in the enamel sections. This, it should be noted, is supported by Andlaw's findings,[14] which were obtained by means of a gravimetric technique. The strong decalcification in the presence of moderate acid production found with orange juice, carbonated beverages, and jam is probably a reflection of the initial acidity of these substances and the consequent longer effect of an acid environment on the enamel. The low decalcification in the presence of high acidity, as with wheat germ, is less easy to explain. The presence in the food of protective factors such as organic phosphates, which will protect the enamel from decalcification, has been suggested by Osborn, Noriskin, and Staz[15] and by Jenkins,[10] and this might account for the discrepancy in decalcification between

wheat flour and wheat germ, but it does not explain other differences, such as those between flour with and without dicalcium phosphate or between the fig cookie and cake or chocolate pudding. In the former instance, we can suggest that a common ion effect slowed the loss of calcium and phosphorus from the enamel. Possibly the cake and pudding exerted a like effect by virtue of ingredients such as milk solids high in phosphate. We believe that this possibility may also account for similar decalcification from sugar and the caramel we used, although the latter produced five times more acid than the former.

Another possibility which may bear on the lack of relationship between acid production and decalcification is that the acids formed may be of different types and, on the basis of Buonocore and Koulouridis' findings,[8, 16] could have widely dissimilar de-

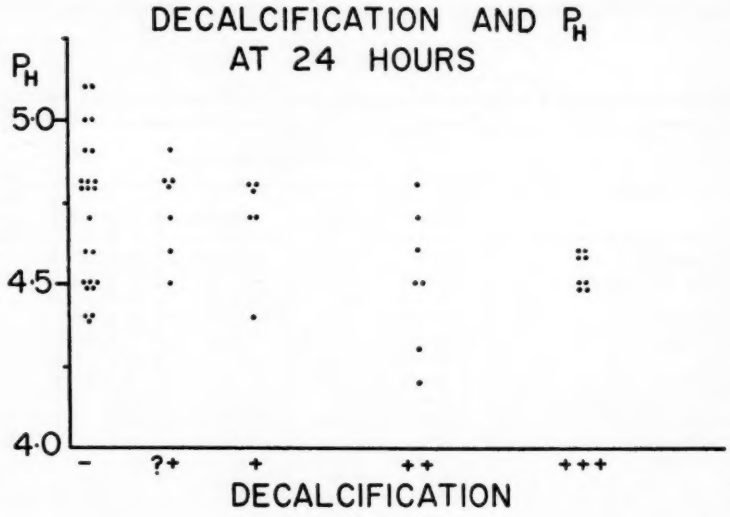


FIG. 1

calcifying properties. Although the buffer capacity of the foods may be responsible, an indication that different acids might be formed during fermentation can be seen in the extreme lack of relationship in the findings for pH and titratable acidity between foods such as wheat flour and wheat germ.

It should be pointed out that the sweet foods, as a whole, produced less acid than did the cereal products. This can be related to the absence of buffers in the former or their initial acidity, which, in either case, quickly gave rise to acid conditions which inhibited further fermentation. Beyond this, it seems that some foods ferment more rapidly than others, and it is interesting to note that, under the test conditions used, natural sugars, such as honey and maple syrup, fermented with particular rapidity. A fuller understanding is needed of the reasons for the wide differences in acid production found for the different foods.

Finally, it can be claimed that measurements of tooth decalcification by foodstuffs have advantages over methods previously used for differentiating between the caries-

producing potentials of foodstuffs and that if work of this sort is expanded and fully interpreted, it could lead to a more satisfactory understanding of the food-caries relationship.

SUMMARY

Polarized light was used to detect changes in form birefringences in "windows" in enamel sections incubated in thirty different food-saliva mixtures. The sections were examined for decalcification at 6, 8, and 24 hours. pH determinations were made at 6 and 8 hours; and both pH and titratable acidity were measured at 24 hours.

The extent of decalcification did not parallel the amount of acid formed. High acid production often accompanied low decalcification, particularly in the presence of wheat germ, dicalcium phosphate, and milk products. Reasons for the findings are offered. It is suggested that the findings may have broad significance in differentiating between the caries-producing capacities of foodstuffs.

REFERENCES

1. Lundquist, C. O. Oral Sugar Clearance, *Odont. Rev.*, Vol. **3**, Suppl. 1, 1952.
2. Ludwig, T. G., and Bibby, B. G. Further Observations on the Caries-producing Potentialities of Various Foodstuffs, *J. D. Res.*, **36**:61, 1957.
3. Caldwell, R. C. Food Retention in the Mouth and Acid Production in Dental Cavities. Thesis, University of Rochester, 1956.
4. Ludwig, T. G., and Bibby, B. G. Acid Production from Different Carbohydrate Foods in Plaque and Saliva, *J. D. Res.*, **36**:56, 1957.
5. Haggard, H. W., and Greenberg, L. A. The Acidity of Saliva after Ingestion of Carbohydrate and Acid-containing Substances, *J. D. Res.*, **30**:126, 1951.
6. Bibby, B. G., Goldberg, H. J. V., and Chen, E. Evaluation of Caries-producing Potentialities of Various Foodstuffs, *J.A.D.A.*, **42**:491, 1951.
7. Buonocore, M. G., and Somner, B. Effect of the Addition of Certain Amino Acids to Enamel Decalcifying Buffers, *J.A.D.A.*, **55**:781, 1957.
8. Buonocore, M. G., and Allison, H. D. Comparative Decalcifying Ability of pH4 Buffers on Enamel and Dentin, *I.A.D.R.*, **35**:92 (abstr.), 1957.
9. Jenkins, G. N., Forster, M. G., Spiers, R. L., and Kleinberg, J. The Influence of the Refinement of Carbohydrates on Their Cariogenicity, *Brit. D. J.*, **106**:195, 1959.
10. ———. The Influence of the Refinement of Carbohydrates on Their Cariogenicity, *ibid.*, p. 362.
11. Stralfors, A. Karieshamning Genom Fosfater. *Svensk. Tandl. Tdskr.*, **49**:108, 1956.
12. McClure, F. J., and Muller, A. Caries Inhibiting Effect of Dibasic Sodium Phosphate and Dibasic Calcium Phosphate Added to Wheat Flour and Bread Diets, *J.A.D.A.*, **58**:36, 1959.
13. Nizel, A. E., and Harris, R. S. Effect of Dietary Levels of Metaphosphoric Acid on Hamster Caries, *J. D. Res.*, **38**:686, 1959.
14. Andlaw, R. Acid Production and Enamel Decalcification by Fermenting Foodstuffs, *J. D. Res.*, **39**:1200, 1960.
15. Osborn, T. W. B., Noriskin, J. N., and Staz, J. A Comparison of Refined Sugar and Cereals in Their Ability To Produce in Vitro Decalcification of Teeth, *J. D. Res.*, **16**:165, 1937.
16. Koulouridis, T. J. The Effect of Organic Anions on Enamel and Dentin Solubility in Acid Buffers, M.S. thesis, University of Rochester, 1958.

Comparative Hydrolysis of Certain Dental Local Anesthetics by Human Serum and Plasma

CHARLES E. BECKER
American Dental Association, Chicago, Illinois

Kalow[1] has presented evidence that procainesterase and human plasma cholinesterase are identical. Foldes, Davis, and Shanor,[2] however, found no parallelism between the hydrolysis rates of acetylcholine, benzoylcholine, succinylcholine, and procaine by human plasma and Cholase, a purified human plasma cholinesterase. These workers and Van Hees[3] determined reaction rate and Michaelis constants of procaine, tetracaine, 4-aminobenzoate, and the corresponding halogenated analogues in plasma and in Cholase. In view of the discrepancies observed in the results obtained in the latter two studies, Foldes, Davis, Shanor, and Van Hees[3] believed the possibilities to be considered included the following:

(1) That more than one enzyme capable of hydrolyzing ACh is present in human plasma and that the activity of these different enzymes toward other esters hydrolyzed by human plasma is variable. It is furthermore conceivable that with the method of purification used in the preparation of Cholase relatively less of the fraction primarily responsible for the hydrolysis of p-ABA esters is extracted. (2) That there is only one plasma cholinesterase but the activity of this one enzyme toward various substrates is affected to a variable degree by the extraction process and/or storage.

This present report is concerned with the enzymatic degradation of certain dental local anesthetics. The comparative hydrolysis of acetylcholine metabutethamine, meprylcaine, piperocaine, procaine, and butethamine by human serum and plasma was studied. There is an apparent over-all parallelism between the rate of hydrolysis of acetylcholine and the ester-type local anesthetics by serum. In addition, divergent results obtained with respect to the effect of anticoagulants and of time suggest the existence of several cholinesterases having variable activity in regard to the hydrolysis of the ester-type local anesthetics.

MATERIALS AND METHODS

Blood samples.—When freshly separated plasma or serum could not be used immediately, the samples were tubed in small aliquots and stored at −15° C. Frozen samples were thawed at 37° C. before use.

Cholinesterase assays.—These determinations were made in quintuplicate by the photometric method of Caraway[4] and in duplicate by the potentiometric method of Michel.[5]

Preliminary reports have been given (*Federation Proc.*, **18**:188, 1959, and **19**:273, 1960).

This work was supported in part by Research Grant D-533 from the N.I.H., U.S. Public Health Service, to Dr. J. Roy Doty, Secretary, Council on Dental Therapeutics, American Dental Association.

Received for publication June 8, 1960.

Incubation.—Serum and substrate were incubated in 0.05 M phosphate buffer, pH 7.4, for 1 hour at 37° C. Serum volumes, substrate concentrations, and final volumes of incubation mixtures were 1 ml., 3.5×10^{-3} M, and 22 ml. for metabutethamine; 1 ml., 2.3×10^{-3} M, and 22 ml. for meprylcaine; 2.5 ml., 1.7×10^{-3} M, and 27.5 ml. for piperocaine; and 2.5 ml., 2.3×10^{-4} M, and 27.5 ml. for both procaine and butethamine. With sera of normal cholinesterase activity, the above concentrations of substrates were sufficient for maximum total hydrolysis during the hour's incubation.

Organic extracts.—Aliquots of the incubation mixtures were diluted with the phosphate buffer to the same final volume as the original incubation mixtures. The pH was adjusted to 8.6 with 0.2 N NaOH. These mixtures were then extracted with equivalent volumes of ethylene dichloride except in the case of metabutethamine, where chloroform was used.

Acid extracts.—The organic extracts were washed twice with equivalent volumes of 0.05 M borate buffer, pH 8.6, and then shaken with 0.1 N HCl or H_2SO_4. The amount

TABLE 1

μM SUBSTRATE HYDROLYZED

(pH 7.4, Ml. of Serum, Hour at 37° C.)

SUBSTRATE	SAMPLES						
	1*	2*	3*	4*	5*	6*	7†
Acetylcholine Cl.‡	115	124	128	134	145	149	180
Metabutethamine HCl	24.1	26.5	26.8	26.2	.....	26.9	38.6
Meprylcaine HCl	12.8	13.5	14.9	15.8	.....	16.9	19.5
Piperocaine HCl	.....	.....	.....	6.1	6.8	6.9	5.9
Procaine HCl	.....	.....	.....	0.75	0.83	0.89	1.3
Butethamine HCl	.....	0.46	.....	.....	0.51	0.53	0.7

* Pooled sample. † Individual sample. ‡ Caraway (see ref. 4).

of metabutethamine recovered in the acid extract was determined by diazotization and coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride according to Bratton and Marshall.[6] The other local anesthetics were determined by ultraviolet spectrophotometry. In the case of procaine and butethamine, the acid extracts were adjusted to pH 8.6 before the readings were made.

Controls.—The specificity of the methods used was evaluated by the distribution technique of Brodie and Udenfriend[7] and found to be satisfactory. Controls were run and corrections were made in each experiment for the amount of spontaneous hydrolysis and the recovery of known amounts of anesthetic.

RESULTS AND DISCUSSION

The results shown in Table 1 are typical and represent the average of replicate determinations. Each pool was made up of serum from 20 to 30 male and female patients hospitalized for medical and surgical reasons. The individual sample is from a normal male.

When the amount of any one anesthetic hydrolyzed is compared from sample to sample, there is an apparent parallelism between the amount of acetylcholine and the

amount of that anesthetic hydrolyzed by the particular sample. And when the amount of procaine hydrolyzed is set to equal 1, the relative ratios of the amounts of the other anesthetics hydrolyzed in comparison with procaine are generally similar from sample to sample. For example, metabutethamine is hydrolyzed about 30 to 35, meprylcaine 15 to 21, piperocaine 5 to 8, and butethamine 0.5 to 0.6 times faster than procaine. In discussing meprylcaine, Monheim[8] states that "Mizzy Co. has attempted to show that it is metabolized in the blood stream fifteen times as rapidly as procaine." Kalow[1] has reported that piperocaine is hydrolyzed about 6 times faster than procaine.

Differences do exist in the ability of oxalated plasma and of serum from the same individual to hydrolyze acetylcholine and the ester-type local anesthetics. In the case of sample No. 7, reading down from acetylcholine through butethamine, values found for oxalated plasma were 68, 82, 93, 98, 70, and 100 per cent, respectively, of the serum values shown in Table 1. In 1943, Kisch, Koster, and Strauss[9] reported that human serum can hydrolyze about 1.4 times as much procaine as citrated plasma. How-

TABLE 2

HYDROLYSIS OF ACETYLCHOLINE CL*

(pH 8.0, Serum, Hour at 37° C.)

Date	Caraway (μM/Ml)	Michel (ΔpH/0.02 Ml)	Date	Caraway (μM/Ml)	Michel (ΔpH/0.02 Ml)
1958			1959		
June–Oct.....	250±7† (239 to 267)‡	1.21	Dec. 18.....	170	.....
1959			1960		
			Feb. 19.....	146	1.39
Sept. 24.....	180	.....	Feb. 23.....	180	1.50
Sept. 29.....	179	.....	Feb. 26.....	169	1.36

* Samples collected from the same individual.
† Mean.
‡ Range.

ever, several workers have assumed that, regardless of the anticoagulants and the methods used, plasma and serum could be interchanged in determining cholinesterase activity. The results of comparative experiments show that, with the method of Michel,[5] oxalated and heparinized, but not citrated, plasmas yield the serum value for cholinesterase. Jorgensen[10] has reported similar findings with a titrimetric method for cholinesterase. With the method of Caraway,[4] heparinized, but not oxalated or citrated, plasma gives the serum value for cholinesterase. Stubbs and Fales[11] used a micromodification of Michel's method and found that oxalate and citrate, but not heparin, inhibit cholinesterase.

During June to October, 1958, ten separate samples of serum were collected from a normal individual at 2-week intervals (Table 2). As measured by the method of Caraway,[4] the serum cholinesterase activity of these samples showed little change during this period. The literature records the fact that, while serum cholinesterase activity may vary widely among different normal individuals, the level in any one individual tends to remain relatively constant. It was found also that the ability of these serum samples to hydrolyze procaine and butethamine showed little change

during these 5 months. Expressed in terms of μM substrate hydrolyzed per ml. of serum per hour at 37° C., the mean value for procaine was 1.66 ± 0.04 (range 1.59–1.74), and for butethamine it was 0.84 ± 0.02 (range 0.77–0.88).

During the 21 months from June, 1958, to February, 1960, serum cholinesterase activity in this individual, as measured by Michel's method,[5] remained at the upper limit of normal of the method, whereas, when measured by the method of Caraway,[4] serum cholinesterase activity showed a decline from the upper limit of normal to the lower limit of normal of the method. The divergent results obtained with the two methods in regard to the effect of anticoagulants and of time suggest that, while both methods measure the amount of acetylcholine hydrolyzed, the two methods do not necessarily measure only the effect of the same enzyme. In the serum of this individual, the apparent decrease in ability to hydrolyze acetylcholine as measured by Caraway's method was reflected in a corresponding decrease in ability to hydrolyze procaine and butethamine as measured by ultraviolet spectrophotometry. In February, 1960, 1 ml. of serum from this individual hydrolyzed 1.25 μM of procaine and 0.67 μM of butethamine after 1 hour's incubation at 37° C.

SUMMARY

The results of comparative experiments with human serum show that acetylcholine is hydrolyzed about 138 to 179, metabutethamine 30 to 35, meprylcaine 15 to 21, piperocaine 5 to 8, and butethamine 0.5 to 0.6 times faster than procaine. An apparent over-all parallelism exists between the rate of hydrolysis of acetylcholine and of the ester-type local anesthetics. In addition, divergent results obtained with respect to the effect of anticoagulants and of time suggest the existence of several cholinesterases having variable activity in regard to the hydrolysis of the ester-type local anesthetics.

The help of Dr. Edward J. Fitzsimons, of Chicago Wesley Memorial Hospital, in securing the blood samples is gratefully acknowledged. Samples of the local anesthetics employed were generously supplied as the hydrochlorides by their manufacturers: metabutethamine (2-isobutylaminoethyl *m*-aminobenzoate) by Novocol Chemical Manufacturing Co., Inc.; meprylcaine (2-methyl-2-propylaminopropyl benzoate) by Oradent Chemical Co., Inc.; piperocaine [3-(2-methyl-1-piperidyl)-propyl benzoate] by Eli Lilly and Co.; procaine (2-diethylaminoethyl *p*-aminobenzoate) by Abbott Laboratories; and butethamine (2-isobutyl-aminoethyl *p*-aminobenzoate) by Graham Chemical Corp.

REFERENCES

1. Kalow, W. Hydrolysis of Local Anesthetics by Human Serum Cholinesterase, *J. Pharmacol. Exptl. Therap.*, **104**:122, 1952.
2. Foldes, F. F., Davis, D. L., and Shanor, S. P. Comparison of Hydrolysis of Acetylcholine, Benzoylcholine, Succinylcholine, and Procaine in Human Plasma, *Federation Proc.*, **13**:354, 1954.
3. Foldes, F. F., Davis, D. L., Shanor, S., and Van Hees, G. Hydrolysis of Ester-Type Local Anesthetics and Their Halogenated Analogues by Purified Plasma Cholinesterase, *J. Am. Chem. Soc.*, **77**:5149, 1955.
4. Caraway, W. T. Photometric Determination of Serum Cholinesterase Activity, *Am. J. Clin. Pathol.*, **26**:945, 1956.
5. Michel, H. O. An Electrometric Method for the Determination of Red Blood Cell and Plasma Cholinesterase Activity, *J. Lab. Clin. Med.*, **34**:1564, 1949.
6. Bratton, A. C., and Marshall, E. K., Jr. A New Coupling Component for Sulfanilamide Determination, *J. Biol. Chem.*, **128**:537, 1939.

7. Brodie, B. B., and Udenfriend, S. The Estimation of Basic Organic Compounds and a Technique for the Appraisal of Specificity, *J. Biol. Chem.*, **158**:705, 1945.
8. Monheim, L. M. *Local Anesthesia and Pain Control in Dental Practice,* chap. 5, p. 150. St. Louis: C. V. Mosby Co., 1957.
9. Kisch, B., Koster, H., and Strauss, E. The Procaine Esterase, *Exptl. Med. Surg.*, **1**:51, 1943.
10. Jorgensen, K. Automatic Recording of Choline Esterase Activities, *Scand. J. Clin. & Lab. Invest.*, **11**:282, 1959.
11. Stubbs, J. L., and Fales, J. T. A Capillary Sampling Technique for the Determination of Cholinesterase Activity in Red Cells and Plasma, *Am. J. Med. Technol.*, **26**:25, 1960.

Electrophoretic Separation of Human Serum Protein That Hydrolyzes Local Anesthetics

CHARLES E. BECKER
American Dental Association, Chicago, Illinois

Marton and Kalow[1] separated human serum protein by paper electrophoresis in barbital buffer. Cholinesterase activity was found to be localized between the α_2- and β-globulin zones by a staining method. However, cholinesterase could not be readily eluted with M/15 phosphate buffer, pH 7.4, from the Whatman No. 1 paper used in the electropherograms.

This present report describes the conditions under which eluates from paper electropherograms of human serum were found to hydrolyze not only benzoylcholine but also acetylcholine and certain ester-type local anesthetics. The results of these experiments suggest the existence of several serum cholinesterases having variable activity in regard to the hydrolysis of the ester-type dental local anesthetics.

EXPERIMENTAL METHODS

Electrophoresis.—Normal human serum (40 μl. per strip) was subjected to paper electrophoresis in a Durrum cell using Schleicher & Schuell 2043A paper and either 0.075 M barbital buffer, pH 8.6, or the 0.5 M Tris (TEB) buffer, pH 8.9, of Aronsson and Gronwall.[2] A constant current of 2.5 m.a. for the barbital buffer and 3.0 m.a. for the TEB buffer was applied for 16 hours at room temperature.

Elution.—Two strips from each run were stained with methanolic bromphenol blue (BPB) to serve as markers for protein. The six remaining unstained strips were dried at 37° C. for 90 minutes. Sections corresponding to zones occupied by albumin and α_1-globulin, α_2-globulin, β-globulin, and γ-globulin were cut from these latter strips. Except for the anode edge of albumin and the cathode edge of γ-globulin, the mid-line between protein fractions served as the boundary line for cutting the sections. The paper sections were in turn cut into small pieces, which were incubated in 0.05 M phosphate buffer, pH 7.4, for 30 minutes at 37° C. When the hydrolysis of acetylcholine was to be measured, the 0.05 M phosphate buffer contained 0.001 per cent phenol red and was of pH 8.0.

Measurement of esterase activity.—The mixtures were clarified by centrifugation, and aliquots of the supernatant fluid were used in place of serum dilutions in measuring the hydrolysis of the following substrates:

1. The hydrolysis of benzoylcholine was measured by the method of Kalow and Lindsay[3] except for an incubation time of 2–3 hours.

Preliminary reports have been given (*Federation Proc.*, **18**:188, 1959, and **19**:273, 1960).

This work was supported in part by Research Grant D-533 from the N.I.H., U.S. Public Health Service, to Dr. J. Roy Doty, Secretary, Council on Dental Therapeutics, American Dental Association.

Received for publication June 8, 1960.

2. The hydrolysis of acetylcholine was measured by the method of Caraway[4] except for an incubation time of 3–4 hours.

3. The hydrolysis of the ester-type local anesthetics was measured as recently described[5] except that substrate concentration was reduced by a factor of 10 and the incubation time was increased to 90 minutes.

Demonstration of enzyme activity on paper.—Serum (8 μl. per strip), Whatman 3MM paper, barbital buffer at 3.6 m.a. for 16 hours, and TEB buffer diluted 1 in 8 at 36v. for 16 hours were used. Wet, unstained strips from each run were cut into two equal, longitudinal sections. One of the sections from each strip was stained with aqueous BPB to serve as a marker for protein. The remaining longitudinal sections were used to demonstrate enzyme activity by placing them in "sandwich" contact with a blank paper strip of similar size wet with the following mixture: 5 ml. each

TABLE 1

LOCALIZATION OF ESTERASE ACTIVITY*

(Barbital Buffer, pH 8.6)

SUBSTRATE	RELATIVE PER CENT			
	Albumin and α_1-Globulin	α_2-Globulin	β-Globulin	γ-Globulin
Benzoylcholine Cl†	0	50	50	0
Acetylcholine Cl‡	0	43	57	0
Metabutethamine HCl	0	77	23	0
Meprylcaine HCl	0	55	46	0
Piperocaine HCl	0	48	52	0
Procaine HCl	0	61	39	0
Butethamine HCl	0	64	36	0

* After elution with 0.05 M phosphate buffer, pH 7.4.
† Kalow and Lindsay (see ref. 3).
‡ Caraway (see ref. 4).

of 0.665 M acetylcholine chloride as substrate and 0.1 per cent phenol red as indicator diluted to a final volume of 25 ml. with 0.05 M phosphate buffer, pH 7.4. The two layers of paper were placed between plastic strips and incubated in a horizontal position for 3 hours at 37° C. Localization of serum cholinesterase activity was revealed by the appearance of a yellow band against the red background of the strips.

RESULTS AND DISCUSSION

As detected by the acetylcholine-phenol red system on the paper strips, with either barbital or TEB buffer, serum cholinesterase was found to be localized between the α_2- and β-globulin zones.

Table 1 shows typical results obtained with serum from the same individual when eluates from the paper electropherograms run with barbital buffer were incubated with benzoylcholine, acetylcholine, and the ester-type local anesthetics as substrates. Results are expressed as the average of replicate determinations. In contrast to the findings of Marton and Kalow,[1] serum cholinesterase could be eluted from the paper;

that is, eluates prepared from the α_2- and β-globulin zones hydrolyzed benzoylcholine. In regard to each of the substrates tested, the approximately equal distribution of activity in the eluates from the α_2- and β-globulin zones is compatible with the presence of esterase activity between those zones.

Similarly, Table 2 shows typical results obtained with serum from the above individual when eluates from the paper electropherograms run with TEB buffer were incubated with benzoylcholine, acetylcholine, and the ester-type local anesthetics as substrates. When compared with the results obtained with the barbital buffer, there is an apparent displacement of esterase activity from between the α_2- and β-globulin zones toward the anode. This displacement is greatest for metabutethamine, meprylcaine, butethamine, and piperocaine and least for benzoylcholine, acetylcholine, and procaine.

TABLE 2

LOCALIZATION OF ESTERASE ACTIVITY*
(TEB Buffer, pH 8.9)

SUBSTRATE	RELATIVE PER CENT			
	Albumin and α_1-Globulin	α_2-Globulin	β-Globulin	γ-Globulin
Benzoylcholine Cl†	0	100	0	0
Acetylcholine Cl‡	0	94	7	0
Metabutethamine HCl	21	66	13	0
Meprylcaine HCl	22	72	6	0
Piperocaine HCl	7	82	11	0
Procaine HCl	0	86	15	0
Butethamine HCl	18	65	18	0

* After elution with 0.05 M phosphate buffer, pH 7.4.
† Kalow and Lindsay (ref. 3).
‡ Caraway (ref. 4).

On the average, with both barbital and TEB buffers, a total of about 48 per cent of the activity present in the serum samples before electrophoresis was recovered in the eluates of the protein zones. Increasing the elution time to longer than 30 minutes did not result in increased recovery of activity.

The results of these experiments suggest the existence of several serum cholinesterases having variable activity in regard to the hydrolysis of the ester-type dental local anesthetics. Dubbs, Vivonia, and Hilburn[6] have recently reported evidence for the subfractionation by starch gel electrophoresis of human serum cholinesterase.

SUMMARY

Paper electrophoresis of normal human serum was carried out in barbital buffer and in the TEB buffer of Aronsson and Gronwall.[2] The comparative localization of esterase activity toward benzoylcholine, acetylcholine, metabutethamine, meprylcaine, piperocaine, procaine, and butethamine was determined. The results of these experiments suggest the existence of several serum cholinesterases having variable activity in regard to the hydrolysis of the ester-type dental local anesthetics.

The help of Dr. Edward J. Fitzsimons, of Chicago Wesley Memorial Hospital, in securing the blood samples is gratefully acknowledged. Samples of the local anesthetics employed were generously supplied as the hydrochlorides by their manufacturers: metabutethamine (2-isobutylaminoethyl *m*-aminobenzoate) by Novocol Chemical Manufacturing Co., Inc.; meprylcaine (2-methyl-2-propylaminopropyl benzoate) by Oradent Chemical Co., Inc.; piperocaine [3-(2-methyl-1-piperidyl)-propyl benzoate] by Eli Lilly and Co.; procaine (2-diethylaminoethyl *p*-aminobenzoate) by Abbott Laboratories; and butethamine (2-isobutylaminoethyl *p*-aminobenzoate) by Graham Chemical Corp.

REFERENCES

1. MARTON, A., and KALOW, W. Studies on Aromatic Esterase and Cholinesterase of Human Serum, *Can. J. Biochem. and Physiol.*, **37**:1367, 1959.
2. ARONSSON, T., and GRONWALL, A. Improved Separation of Serum Proteins in Paper Electrophoresis —a New Electrophoresis Buffer, *Scand. J. Clin. & Lab. Invest.*, **9**:338, 1957.
3. KALOW, W.. and LINDSAY, H. A. A Comparison of Optical and Manometric Methods for the Assay of Human Serum Cholinesterase, *Can. J. Biochem. and Physiol.*, **33**:568, 1955.
4. CARAWAY, W. T. Photometric Determination of Serum Cholinesterase Activity, *Am. J. Clin. Pathol.*, **26**:945, 1956.
5. BECKER, C. E. Comparative Hydrolysis of Certain Dental Local Anesthetics by Human Serum and Plasma, *J. D. Res.*, **40**:190, 1961.
6. DUBBS, C. A., VIVONIA, C., and HILBURN, J. M. Subfractionation of Human Serum Enzymes, *Science*, **131**:1529, 1960.

Carbonyl-binding Compounds as Inhibitors of Experimental Caries in Rats

HAROLD V. JORDAN, ROBERT J. FITZGERALD, and NORMA D. BERGER

National Institute of Dental Research, National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare, Bethesda, Maryland

The use of agents which inhibit or otherwise adversely influence the normal glycolytic sequence in oral bacteria is generally considered to be a rational approach to the caries-control problem.[1] One such compound, sodium metabisulfite, has recently been reported to inhibit experimental caries in rats.[2] Inhibition of the growth of a variety of micro-organisms was also noted. This compound has been widely employed as a complexing agent for aldehydes and methyl ketones, particularly the metabolic intermediates, acetaldehyde and pyruvate.[3–5] These observations have prompted the study of two other carbonyl-binding compounds. One of these agents, carboxylmethoxylamine hemihydrochloride* (CMA) has been shown to inhibit certain bacteria, presumably by its ability to trap pyruvate.[6] It has also been used for the isolation of small amounts of ketones from unsaponifiables, such as cholesterol and its metabolic products,[7] and for the isolation of higher fatty aldehydes from animal muscle and brain.[8] Dimedone,† the other agent, has been used in studies on microbial metabolism because of its specificity as an aldehyde-trapping reagent.[3, 4]

In the present work the effects of these compounds were investigated in vivo against experimental caries in rats and also in vitro for their effects on the growth of pure cultures of certain micro-organisms.

EXPERIMENTAL METHODS

The procedures for testing the compounds have been previously described.[2] For the anticaries tests the compounds were blended into the coarse corn particle diet 585 described by Stephan, Fitzgerald, McClure, Harris, and Jordan.[9] Diet and distilled water were provided ad libitum to groups of 24 weanling female Sprague-Dawley rats which were randomly distributed by threes in screen-bottom cages. After 90 days the dentition was evaluated for caries of the molar sulci, using the scoring system of Cox, Dodds, Dixon, and Matuschak.[10]

Three separate animal experiments were performed. In the first experiment sodium metabisulfite was incorporated in cariogenic diet 585 at a level of 0.3 per cent. In the second experiment dimedone was mixed in diet 585 at a level of 0.4 per cent. In the third experiment CMA was initially tested at 0.1 per cent, but after the first week

Received for publication July 11, 1960.

* (Aminooxy) acetic acid hemihydrochloride, Eastman Organic Chemicals.

† 5-dimethyl-1,3-cyclohexanedione, Eastman Organic Chemicals.

this was reduced to 0.05 per cent because the animals were not gaining weight at a satisfactory rate. In each experiment an equal number of control animals received unsupplemented diet 585.

The methods used for the antibacterial tests and the inhibition of lactic acid production were the same as those previously described for the studies on sodium metabisulfite.[2] In the antibacterial tests, filter-sterilized stock solutions of the compounds were added aspectically to tubes of the appropriate media, and a series of threefold dilutions was made.[2] Because of its limited solubility, dimedone was put into solution by the addition of a few drops of 10 per cent NaOH to the stock solution.

The micro-organism used for the tests on lactate production was a streptococcus, strain FA-1, which was isolated from the oral cavity of a caries-active rat and which had been shown to induce caries when inoculated orally into germ-free rats.[11] Filter-

TABLE 1

INHIBITION OF SULCAL CARIES IN RATS BY CARBONYL-BINDING COMPOUNDS

	Experiment 1		Experiment 2		Experiment 3	
	Na Metabisulfite	Control	Dimedone	Control	CMA	Control
Conc. in diet 585 (per cent)	0.3	.....	0.4	.....	0.05*	.....
Survivor ratio	22/24	23/24	23/24	23/24	22/24	24/24
Final body wt. (av.)	205.4 gm.	194.8 gm.	187.3 gm.	192.3 gm.	187.8 gm.	202.0 gm.
Daily food intake (av.)	12.3 gm.	9.1 gm.	10.4 gm.	11.5 gm.	9.1 gm.	11.3 gm.
Per cent rats with caries	82	100	100	100	87	100
Carious teeth per rat	2.1	4.3	3.3	4.0	2.2	4.5
Carious surfaces per rat	2.3	9.6	4.6	7.6	2.6	9.6
Caries score per rat	2.8†	20.3	7.1†	15.7	3.6†	20.4
Standard error	±0.7	±1.9	±0.9	±1.8	±0.7	±1.8

* 0.1 per cent for the first week, 0.05 per cent thereafter.

† Each score is significantly different from its control at the 0.01 level.

sterilized solutions of the compounds were tested in a suitable medium containing 1 per cent glucose. The final concentrations of the compounds tested were at or below the minimal growth-inhibiting level. Since CMA was completely inhibitory at 0.21 millimolar, using the medium and inoculum previously described,[2] its effect on lactate production was tested at one-third of this concentration (0.068 millimolar). This concentration had no effect on growth. Dimedone was tested at 0.0714, 0.357, and 0.714 millimolar. There was a very slight inhibition of growth at the highest concentration. After inoculation the cultures were incubated at 37° C. for sufficient time to allow the maximum turbidity and lowest pH to be attained. It is known from previous experiments that these levels are usually reached in 48 hours or less with this organism.

RESULTS

Each of the carbonyl-binding compounds effected a marked reduction in the severity of sulcal caries in rats, as shown in Table 1. Inclusion of 0.3 per cent sodium metabisulfite in the diet resulted in a reduction of 86 per cent in the caries score. This

confirms the previous observation concerning the inhibition of experimental caries by this compound.[2] CMA was also a potent caries inhibitor. A caries reduction of 82 per cent over the control group was obtained by the use of this compound. Dimedone, at a level of 0.4 per cent in the diet, resulted in a 55 per cent reduction in the caries score as compared with its control.

CMA was also found to inhibit the in vitro growth of a variety of micro-organisms. In Table 2 the various genera are arranged according to their relative sensitivities to the reagent. Inhibitory levels are expressed in the table as molar concentrations

TABLE 2

INHIBITION OF SELECTED MICRO-ORGANISMS BY CARBOXYLMETHOXYLAMINE

	Strain No.	Inhibitory Concentration (mM)/l*
Candida pseudotropicalis	360	>4.58†
Candida krusei	379	4.58
Candida krusei	359	4.58
Candida albicans	358	4.58
Candida albicans	TF	4.58
Lactobacillus plantarum	8014	4.58
Lactobacillus arabinosus	17-5	4.58
Lactobacillus casei	7469	4.58
Lactobacillus fermenti	36	1.51
Lactobacillus fermenti (rat)	NR77	1.51
Lactobacillus acidophilus (rat)	42T	0.50
Streptococcus faecalis	SLR	1.51
Streptococcus salivarius	9756	1.51
Streptococcus salivarius	3	1.51
Streptococcus mitis	2	1.51
Streptococcus (rat)	FA-1	1.51
Diphtheroid	D688	1.51
Neisseria catarrhalis	N6	1.51
Neisseria flava	N8	0.50
Leptotrichia	C-118-C	0.50
Leptotrichia	C-148-C	0.50
Staphylococcus aureus	13	0.50
Staphylococcus aureus	33	0.50
Micrococcus	19	0.50
Micrococcus	6	0.17
Gaffkya tetragena	10875	0.17

* First tube in series which showed complete inhibition.
† Corresponds to 0.1 per cent.

for comparison with the other compounds. *Candida* strains were the most resistant, with *Candida pseudotropicalis* not completely inhibited at 0.1 per cent (4.58 millimolar), the highest concentration tested. Three homofermentative lactobacilli were as resistant as the *Candida* strains. The two heterofermentative lactobacilli (*Lactobacillus fermenti*) were of the same order of sensitivity as all the streptococci, a single diphtheroid, and *Neisseria catarrhalis*. *Lactobacillus acidophilus*, two strains of leptotrichiae, two staphylococci, *N. flava*, and a micrococcus displayed a greater degree of sensitivity. *Gaffkya tetragena* and a second micrococcus were the most sensitive organisms tested, being inhibited at a concentration of 0.17 millimolar. The range of inhibitory concentrations reported here compare favorably with those published

by Favour[6] for a different group of organisms, although the yeasts tested by that author were considered to be unaffected by CMA.

In contrast to these results, dimedone was found to be a much weaker inhibitor of bacterial growth in vitro. The same organisms were not completely inhibited at concentrations of dimedone up to 28.6 millimolar, the highest concentration tested, with the single exception of streptococcus strain FA-1, which was inhibited at this concentration.

The inhibitory levels reported here for CMA and dimedone can be compared with previously reported values for inhibition of essentially the same group of organisms by sodium metabisulfite. Effective concentrations ranged from 21.1 millimolar sodium metabisulfite for inhibition of *C. albicans* and certain streptococci down to 0.53 millimolar for inhibition of a leptotrichia.[2]

There was no apparent effect on the amount of lactic acid produced by growing cultures of streptococcus strain FA-1 incubated for 48 hours in the presence of subinhibitory concentrations of either CMA or dimedone. The molar ratio of lactate produced to glucose used was approximately 1.8: 1.0 in the presence of either compound, including the control.

DISCUSSION

When it is considered that the relative concentrations of the three inhibitors fed in the diet were 0.029 moles (0.4 per cent) for dimedone, 0.016 moles (0.3 per cent) for sodium metabisulfite, and between 0.004 and 0.002 moles (0.1–0.05 per cent) for CMA, it seems obvious that the latter compound was the most effective caries-inhibiting agent. This is based on the reasoning that CMA inhibited caries almost to the same extent as did metabisulfite, even though there was initially a fourfold difference and ultimately an eightfold difference in their molar concentrations in the diet. Dimedone was obviously the least effective in this respect. This may be due in part to its relatively lower solubility in comparison with the other two agents tested. A consideration of the stoichiometry of the reactions between these agents and carbonyl-containing compounds also helps to explain why both metabisulfite and CMA would probably be more effective than dimedone on a molar basis. Sodium metabisulfite and carboxylmethoxylamine hemihydrochloride would each be expected to yield 2 moles of carbonyl-reactive compound from 1 mole of the parent compound. Each of these would then react on a mole-for-mole basis with available carbonyl groups.[6, 12] On the other hand, 2 moles of dimedone are required to complex a single mole of available aldehyde.[12] Thus, on a molar basis, CMA and metabisulfite would be theoretically four times more effective than dimedone. In this respect it may also be significant that the two reagents which bind both aldehydes and pyruvate are also the more effective caries-inhibiting agents.

The greater activity of CMA in comparison with sodium metabisulfite is somewhat offset by its greater toxicity[6] and the fact that metabisulfite inhibits lactate production at concentrations below those at which bacterial growth is inhibited.[2] Neither CMA or dimedone shares this property. This may be a reflection of the stability of the different carbonyl complexes, although definitive evidence on this point is lacking.

SUMMARY

Three different carbonyl-binding compounds were found to inhibit the development of sulcal caries in Sprague-Dawley rats when incorporated separately into a coarse corn particle, high-sugar diet. An 82 per cent reduction in caries score was obtained when carboxylemethoxylamine hemihydrochloride was fed in the diet at a level of 0.004 molar for the first week and 0.002 molar for the remainder of the 90-day test period. Feeding of 0.016 molar sodium metabisulfite resulted in an inhibition of 86 per cent. Rats fed the aldehyde-binding agent dimedone at a level of 0.029 molar in the diet showed a 55 per cent reduction in caries score. Relative effectiveness of these three compounds as caries-inhibiting agents generally paralleled their growth-inhibiting properties for a variety of micro-organisms, including lactobacilli, streptococci, staphylococci, micrococci, candidae, leptotrichiae, diphtheroids, and neisseriae. Subinhibitory concentrations of carboxylmethoxylamine hemihydrochloride and dimedone apparently had no effect on lactate production by a rat oral streptococcus, in contrast to earlier observations indicating that subinhibitory concentrations of sodium metabisulfite can depress lactate production by this organism.

REFERENCES

1. Fosdick, L. S. Enzyme Inhibitors as a Factor in the Control of Dental Caries, *J.A.D.A.*, **52**:9, 1956.
2. Jordan, H. V., Fitzgerald, R. J., and Bowler, A. E. Inhibition of Experimental Caries by Sodium Metabisulfite and Its Effect on the Growth and Metabolism of Selected Bacteria, *J. D. Res.*, **39**:116, 1960.
3. Freeman, G. G., and Donald, G. M. S. Fermentation Processes Leading to Glycerol. I. The Influence of Certain Variables on Glycerol Formation in the Presence of Sulfites. II. Studies on the Effects of Sulfites on Viability, Growth and Fermentation of *Saccharomyces cerevisiae*, *Appl. Microbiol.*, **5**:197, 211, 1957.
4. Wood, H. G., Stone, R. W., and Werkman, C. H. The Intermediate Metabolism of the Propionic Bacteria, *Biochem. J.*, **31**:349, 1937.
5. Wallerstein, J. S., and Stern, K. G. On Bisulfite as an Inhibitor of Carboxylase and the Mechanism of Glycerol Fermentation, *J. Biol. Chem.*, **158**:1, 1945.
6. Favour, C. B. Bacteriological Study of Carboxylmethoxylamine Hemihydrochloride, *J. Bacteriol.*, **55**:1, 1948.
7. Anchel, M., and Schoenheimer, R. Reagents for the Isolation of Carbonyl Compounds from Unsaponifiable Material, *J. Biol. Chem.*, **114**:539, 1936.
8. Anchel, M., and Waelsch, H. The Higher Fatty Aldehydes. I. Isolation from Small Amounts of Tissue with Acidic Carbonyl Reagents, *J. Biol. Chem.*, **145**:605, 1942.
9. Stephan, R. M., Fitzgerald, R. J., McClure, F. J., Harris, M. R., and Jordan, H. V. The Comparative Effects of Penicillin Bacitracin Chloromycetin, Aureomycin, and Streptomycin on Experimental Dental Caries and on Certain Oral Bacteria in the Rat, *J. D. Res.*, **31**:421, 1952.
10. Cox, G. J., Dodds, M. L., Dixon, S. F., and Matuschak, M. C. Experimental Dental Caries. III. A System of Recording Cornmeal Caries in Rats, *J. D. Res.*, **18**:469, 1939.
11. Fitzgerald, R. J., Jordan, H. V., and Stanley, H. R. Experimental Caries and Gingival Pathology in the Gnotobiotic Rat, *J. D. Res.*, **39**:923, 1960.
12. Noller, C. R. *Chemistry of Organic Compounds*, p. 736. Philadelphia: W. B. Saunders Co., 1951.

Studies of the Growth of the Upper Facial Skeleton Using Radioactive Calcium

ANDREW D. DIXON

Department of Anatomy, College of Medicine, State University of Iowa, Iowa City

Important studies using a variety of methods have contributed to our knowledge of the growth of the bones of the face and cranium in man and animals. Methods applied to the experimental animal have included those which involve adaptations of the cephalometer,[1, 2] metallic implants,[3] and the feeding or injection of vital dyes.[4–6] Some investigators[7, 8] have employed combinations of these procedures to trace the rate of growth and to determine specific growth sites.

The advent of radioactive isotopes has provided the additional technique of autoradiography for the study of bone growth, and comprehensive works on mineral acquisition by bone have been carried out principally with isotopes of phosphorus[9] or calcium,[10–11] utilizing a variety of autoradiographic procedures.[12] These investigations have been concerned essentially with the growth of long bones or the dynamics of the metabolic changes concerned and make but limited reference to the uptake of radioactive materials by dental tissues.

Radioactive calcium (Ca^{45}) has not been used extensively to study skull growth,[13] and the present investigation was designed to inquire more fully into its usefulness in revealing growth mechanisms in this region, as an elaboration of previous studies of early human jaw development.[14] Particular attention has been directed toward the contributions made by surface deposition, suture growth, and cartilage replacement to the increase in size of the upper facial skeleton and associated cranial bones.

MATERIALS AND METHODS

Thirty-five white rats (Wistar strain) were injected intraperitoneally with 2.5–10 μc. per 100 gm. of body weight of radioactive calcium in the form of an isotonic calcium chloride solution. The age of the animals ranged from birth to 62 days, and in the case of the younger animals it was found difficult to prevent leakage of the isotope from the injection site. Consequently, considerable precautions were necessary to prevent contamination of laboratory equipment and personnel. Several pregnant rats were injected at 1–4 days before the anticipated birth of their litters, to observe the uptake of Ca^{45} by the fetal skull and teeth. No toxic effects of the isotope were observed in any of the animals, and growth curves were normal. A limited number of animals received injections of radioactive phosphate (P^{32}), but this practice was discontinued, as Ca^{45} produced a greater resolution of the autoradiographic image. Animals were killed 30 minutes to 77 days after injection, some animals receiving a second equivalent dose of Ca^{45} 12 hours before death, in an attempt to study increments of bone growth during the experimental period.

Received for publication June 29, 1960.

The soft tissues were removed by dissection from the skulls and teeth in all but the smallest animals. The skulls were dried in an oven and imbedded in paraffin wax or methyl methacrylate. The resulting blocks were ground down on glass paper or a modified watchmaker's lathe to a chosen surface. Serial contact autoradiographs at approximately 250-μ intervals were made from the bones on orthochromatic film in the coronal, sagittal, and horizontal planes. Orthochromatic film, although it required longer exposure times, gave results superior to those obtained on dental X-ray film. Exposure in the dark of the radioactive material to the photographic emulsion varied from 72 to 96 hours.

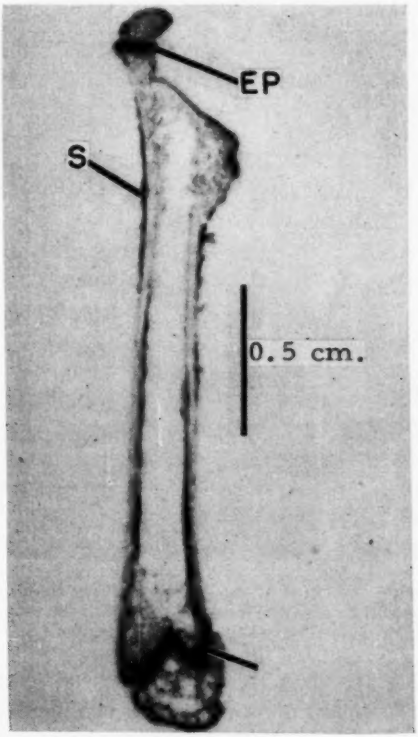


FIG. 1.—Median longitudinal autoradiograph prepared from the femur of a 57-day-old rat which was injected with 10 μc Ca^{45} 12 hours before death. Note the concentration of the isotope in areas of periosteal deposition (*S*) and in the epiphysial plates (*EP*).

RESULTS

To aid in the interpretation of subsequent illustrations, an autoradiograph prepared from the femur of a 50-day-old rat, injected 18 hours before death, is shown in Figure 1. The resolution is such that areas of subperiosteal deposition of radioactive calcium can be observed at the upper end of the shaft of the bone, in contrast to endosteal deposition toward its lower end. These dark linear zones are to be compared with lighter areas which denote regions which have less radioactive material, suggesting that they were less active sites of bone formation during the period of

Ca^{45} uptake. The epiphyseal plates at either end of the bone are intensely radioactive, as would be anticipated from the known importance of these plates as growth centers for the increase in length of long bones.

The uptake of radioactive calcium by bone is extremely rapid (Fig. 2). In this 1-day-old rat, which was injected 30 minutes before death, the bones of the skull are already accurately outlined. The fact that the soft tissues were not removed prior to preparing the autoradiograph provides evidence of the high degree of selectivity of Ca^{45} for calcifying tissues. Individual skull bones can be identified, and the greater uptake of the isotope in the immediate region of the sutures of the cranial base will be apparent.

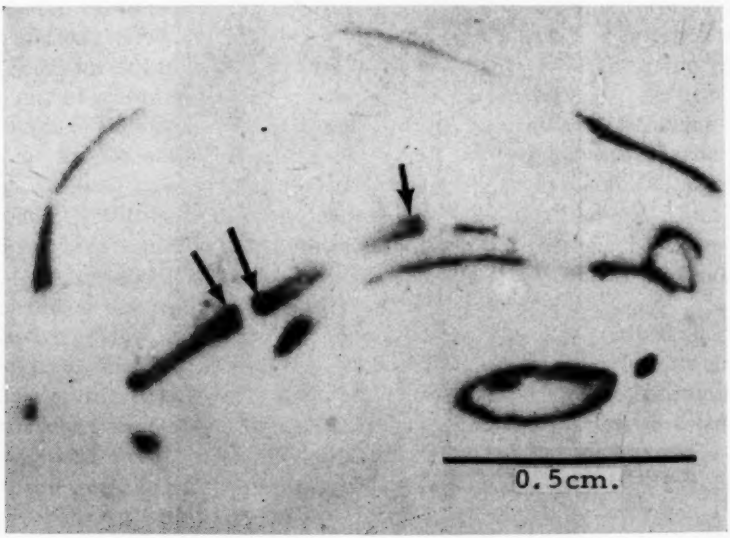


FIG. 2.—Mid-line sagittal autoradiograph of the skull of a rat less than 1 day old. Ca^{45} was injected 30 minutes before death. The isotope has been localized rapidly in the bones. Note the heavier deposition of Ca^{45} (*arrowed*) in immediate relationship to the cartilaginous suture zones in the skull base.

An autoradiograph (Fig. 3) which represents a sagittal section through the lateral part of the skull of a 4-day-old rat reveals that a considerable amount of the Ca^{45} which was injected into the mother 2 days before the birth of the animal has been transferred across the placenta to be utilized by the fetal bones and developing dental tissues. Apart from a general increase in the dimensions of the skull bones, which has resulted in a reduction in the size of sutural intervals, it will be noted that the cusps of the cheek teeth are undergoing calcification. Histologic control sections show that the radioactive material is confined to the developing dentin within the tooth germs at this stage.

Horizontal plane autoradiographs (Fig. 4) through the cranial base and maxillary region of older animals demonstrate several features of skull growth. Areas of surface deposition of Ca^{45} on the outer surface of the maxillae are in contrast to areas of relative inactivity on the medial aspects of the bones. Intense radioactivity is evident at a mid-line cranial base synchondrosis which contributes to increase in length of

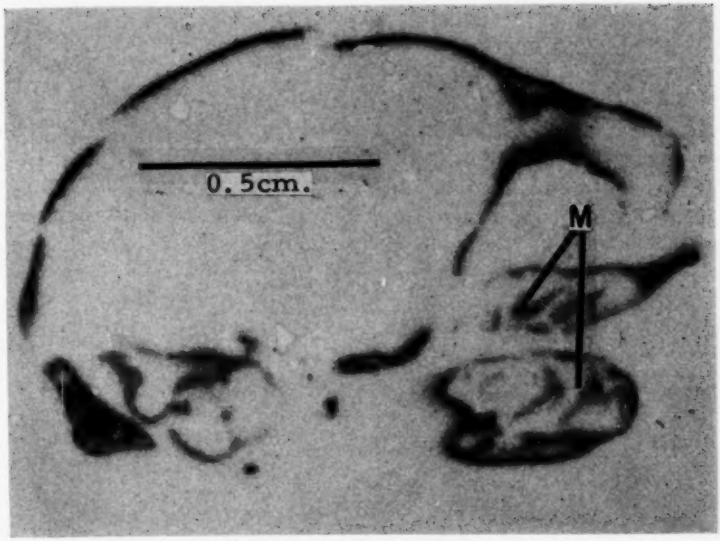


FIG. 3.—Para-sagittal autoradiograph of the skull of a rat killed 4 days after birth. The mother had been injected with 10 μc Ca^{45} 2 days before birth of the litter. The isotope has entered the fetus via the placenta, to be utilized by the developing bones and molar teeth (*M*).

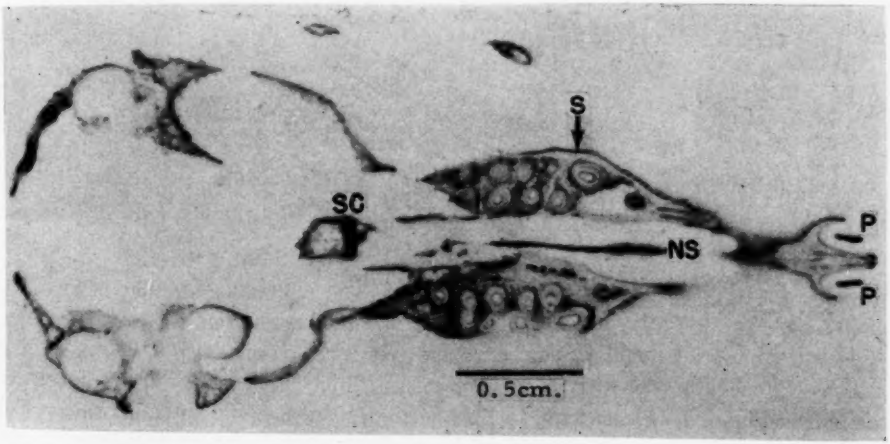


FIG. 4.—Autoradiograph in the horizontal plane through the cranium and upper jaw of a 57-day-old rat injected 18 hours before death. Note the intense radioactivity of the cranial base synchondrosis (*SC*), the nasal septum (*NS*), and the pulps of the incisors (*P*). Areas of surface deposition (*S*) occur on the outer aspect of the maxillae.

the skull between the foramen magnum and the incisor teeth. The cartilage of the nasal septum is also heavily outlined. Within the upper jaw, patterns of concentric rings, indicating discrete laminae of Ca^{45} uptake, represent transverse sections through the roots and sockets of the molar teeth.

Figure 5 presents the maxillary region of Figure 4 in greater detail, to show more clearly the manner of Ca^{45} deposition in relationship to the roots of the molar teeth. In several instances three concentric rings can be identified, and this is particularly clear in the case of the roots of the first molar tooth. From within outward, these rings represent radioactivity in the predentin layer, the layer of calcifying cementum, and the lamina dura of the tooth socket. It will be noted that deposition of Ca^{45}

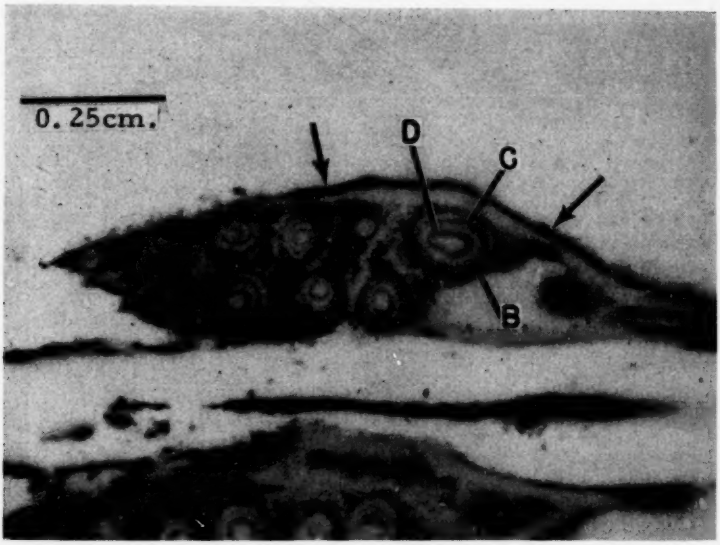


FIG. 5.—Higher magnification of the roots of the cheek teeth shown in the previous figure. Note the concentration of Ca^{45} on the buccal aspect of the maxilla (*arrowed*) and the concentric rings in the region of the molar roots corresponding to the predentine zone (*D*), cementum (*C*), and bone (*B*) lining the dental socket.

is more pronounced on the lingual aspect of the anterior root of the first molar than on its buccal side. A histologic control from a littermate, sectioned in the same plane, demonstrates these zones in a routine preparation (Fig. 6).

A higher magnification of the synchondrosis (Fig. 7), which occupies a central position in Figure 4, reveals that Ca^{45} has been deposited in the form of two parallel bands. On either side of these bands of intense radioactivity, fainter, irregularly disposed striations indicate the trabecular pattern of newly formed bone produced in response to cartilaginous replacement. Histologic controls (Fig. 8) suggest that the zones of high Ca^{45} activity correspond to the regions of calcifying cartilage on either side of the central proliferating cartilaginous plate. Autoradiographs prepared in the coronal plane through the same region (Fig. 9) demonstrate a disklike form of isotope accumulation in the synchondrosis, implying a greater growth potential when compared with the neighboring tympanic bullae and cranial vault. A prepara-

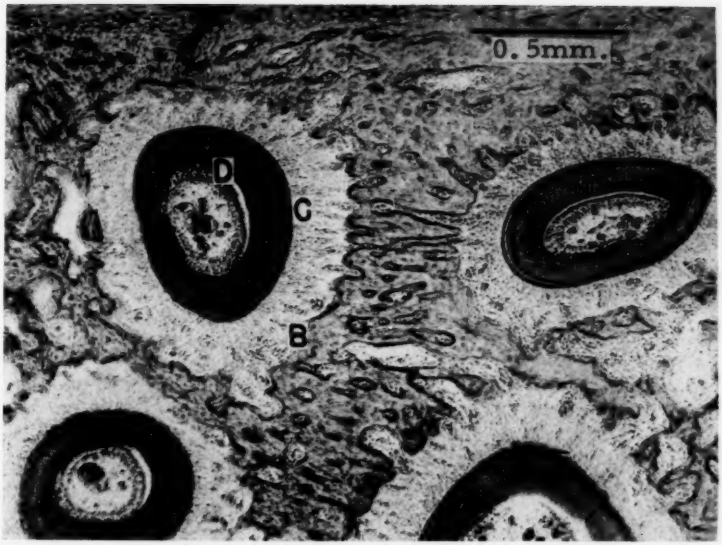


FIG. 6.—Histologic control for the autoradiograph shown in Fig. 5. The labeling is the same as in the previous figure. Hemtatoxylin and eosin. ×54.

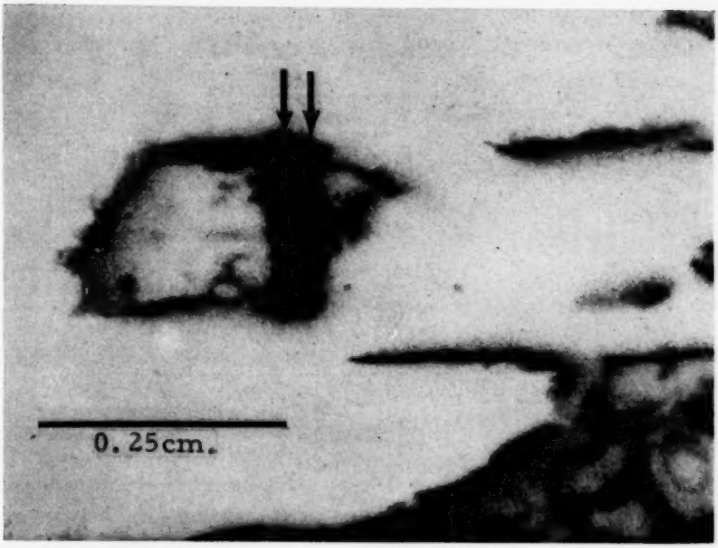


FIG. 7.—Higher magnification of the synchondrosis in the cranial base shown in Fig. 4. The granular appearance is due entirely to the nature of the beta-particle emission from the isotope. Note the two bands of high radioactivity (*arrowed*) in the suture zone.

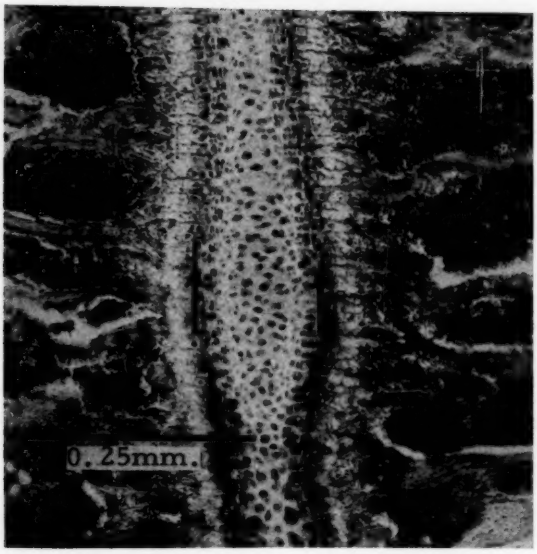


FIG. 8.—Histologic control from a littermate for the features shown in Fig. 7. The corresponding zones are arrowed and are the regions of calcifying cartilage in the suture. Hematoxylin and eosin. ×135.

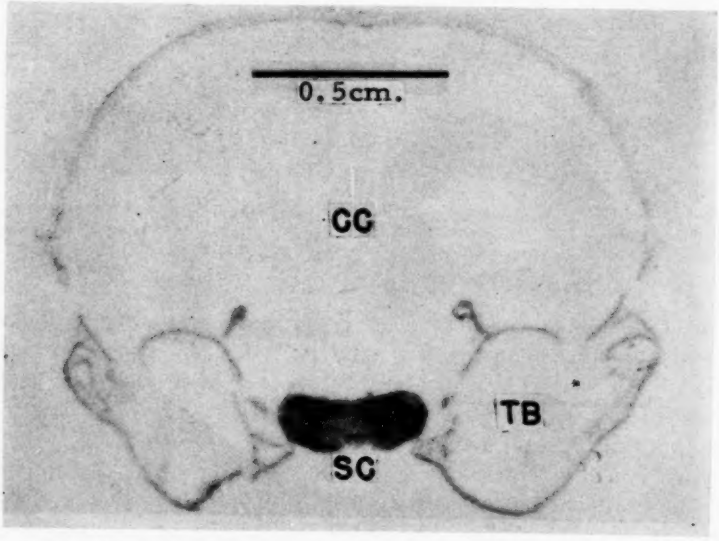


FIG. 9.—Dense ovoid area (*SC*) indicating the spheno-occipital synchondrosis in a coronal plane autoradiograph. The radioactivity of this area should be contrasted with sites of less rapid growth in surrounding bones. *TB*, tympanic bulla; *CC*, cranial cavity.

tion from an area anterior to the synchondrosis (Fig. 10) is to be compared with Figure 9 to emphasize the decrease in radioactivity beyond the suture area.

Sutural activity and surface deposition of Ca^{45} can be shown in other situations by means of autoradiographs at various levels and in alternative planes. Figure 11 is in a series with a similar preparation, already described (Fig. 4). At this lower level the mid-palatal suture is indicated by a dark band of radioactivity, while surface deposition of Ca^{45} is observed on the lingual aspect of the molars toward the lower margin of the photograph. Between these areas the plane of the autoradiograph passes through the horizontal process of the maxilla, and the heavy curved lines record the presence of Ca^{45} on its inferior, palatal surface. Because of the palatal curvatures, a single autoradiograph does not show the latter deposition throughout the entire area of the hard palate.

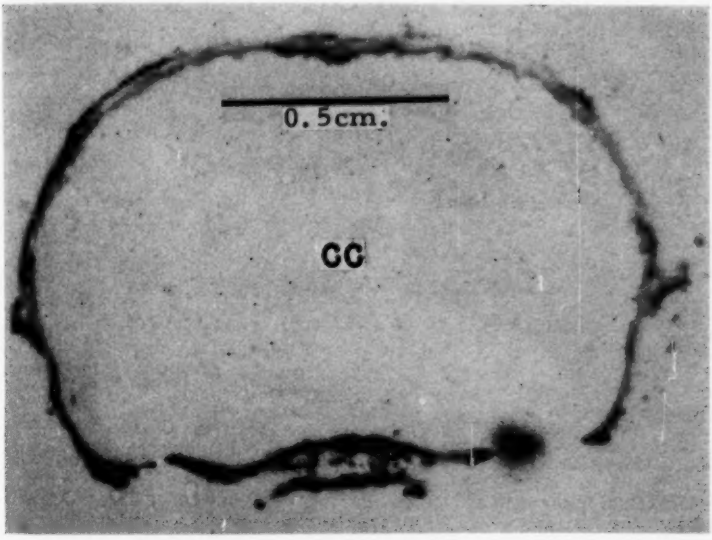


FIG. 10.—An autoradiograph from a more anterior plane in the skull from which Fig. 9 was prepared. The decrease in radioactivity in the mid-basal region of the skull is evident. *CC*, cranial cavity.

Activity of facial sutures can be observed in Figure 12, which is a horizontal autoradiograph through the facial skeleton and cranial vault above the level of the roots of the teeth. It is to be noted that the zygomatico-maxillary and premaxilla-maxillary sutures, prominently displayed by the high content of Ca^{45} within them, are similarly inclined on each side of the upper jaw. They are suitably directed, to allow for forward and outward growth of the facial bones in response to surface deposition and growth of the nasal septal zone. Because this 60-day-old animal was allowed to survive for a period of 14 days following injection of Ca^{45}, the zygomatico-maxillary suture in particular is not so sharply defined as would be the case in animals of short survival periods. A central dense zone in the suture marks the site of Ca^{45} uptake immediately following its injection. The lighter zone which fades away

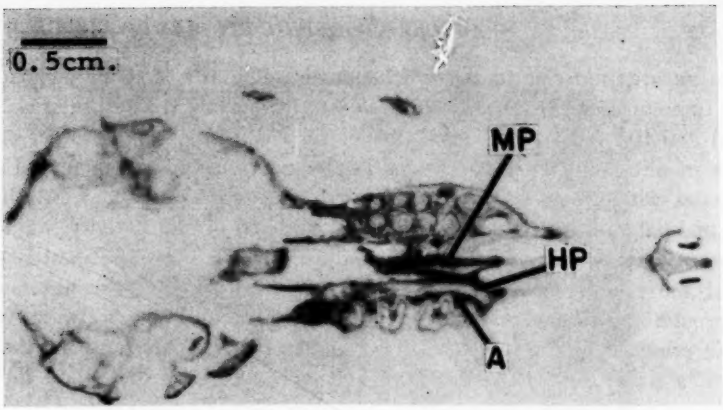


FIG. 11.—Horizontal plane autoradiograph from the skull of a 57-day-old rat showing activity at the mid-palatal suture (*MP*). *A*, surface deposition of Ca^{45} at the alveolar margin; *HP*, deposition on the inferior surface of the hard palate.

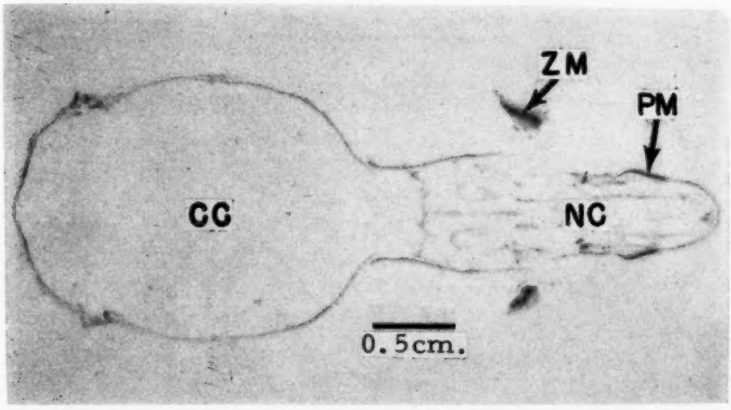


FIG. 12.—Horizontal plane autoradiograph showing Ca^{45} uptake at the premaxilla-maxillary (*PM*) and zygomatico-maxillary (*ZM*) sutures in relation to facial growth. Sixty-day rat. Fourteen days' survival after injection of Ca^{45}. *NC*, nasal cavity; *CC*, cranial cavity.

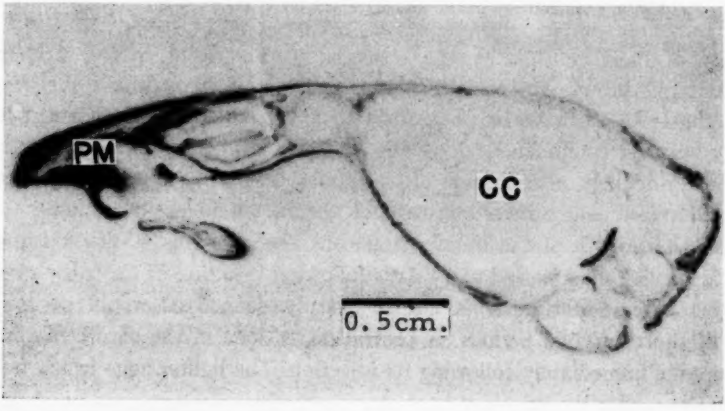


FIG. 13.—Intensive deposition of Ca^{45} on the facial aspect of the premaxilla in a 60-day-old rat. Eighteen days' survival after injection. *CC*, cranial cavity; *PM*, premaxilla.

on either side of this darker band traces the continued deposition of Ca^{45} throughout succeeding days before the death of the animal.

Sagittal plane autoradiographs (Figs. 13 and 14) demonstrate surface deposition on the outer surface of the premaxilla and the alveolar margin, respectively. The high level of radioactivity is a striking feature in the premaxillary region and can be correlated with the rapid increase in length of the snout of the rat, which occurs at this time. Alveolar deposition, most apparent between the roots of the molar teeth, is seen to better advantage in Figure 15. This coronal plane preparation emphasizes

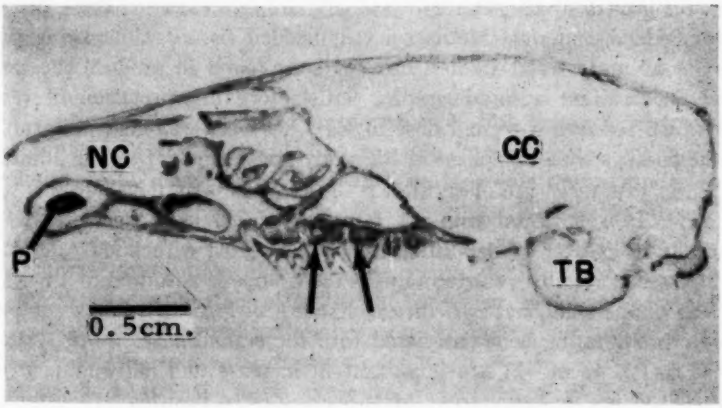


FIG. 14.—Uptake of Ca^{45} by developing bone at the alveolar margin in a 60-day-old rat. *CC*, cranial cavity; *TB*, tympanic bulla; *NC*, nasal cavity; *P*, pulp of incisor tooth. Arrows indicate alveolar deposition.

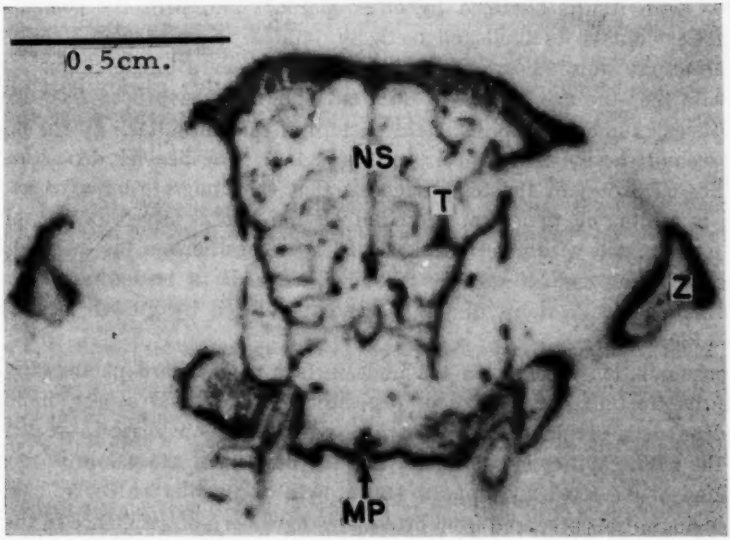


FIG. 15.—Deposition of Ca^{45} on the undersurface of the hard palate and alveolar margin. Coronal autoradiograph prepared from a 50-day-old animal. Survival period 4 days after injection. *NS*, nasal septum; *T*, nasal conchae; *Z*, zygomatic arch; *MP*, mid-palatal suture.

the nasal area, including the nasal septum and the delicate tracery formed by Ca^{45} accumulation within the nasal conchae. Alveolar deposition of Ca^{45} can be traced medially into continuity with a similar pattern on the undersurface of the hard palate and within the mid-palatal suture. In contrast, little Ca^{45} has been taken up on the nasal surface of the hard palate, so that it is difficult to determine the full thickness of the bone in some regions.

DISCUSSION

The autoradiographs which form the basis of the present study represent various stages in the growth of the skull and jaws and are selected from those prepared from paraffin-imbedded material. Methacrylate-imbedded tissue, while being particularly suitable for accurate serial studies, has not been found to produce the same clarity of detail on resultant autoradiographs. Satisfactory interpretation of results from animals which received a second dose of Ca^{45} has been extremely difficult. For this reason the findings from this phase of the study are withheld, pending additional data.

Ca^{45} is an extremely useful substance for the study of facial growth and dental development. It is localized with great rapidity in growing bones, and qualitative differences in its pattern of deposition can be correlated with known facts about jaw growth at various ages. In younger animals the isotope is adsorbed to a marked degree in all areas of the skull, but even in early stages of growth it has a predilection for the rapidly proliferating bone associated with the cranial base suture systems. These sutures (Figs. 2, 4, and 7) are important in increase of skull and face length. At later stages of jaw growth, areas of subperiosteal deposition are established and contribute to the increase in width and depth of the upper jaw.

The most pronounced deposition of Ca^{45} has been noted at all ages in both fibrous and cartilaginous sutures and to the greatest extent in younger animals. This is in agreement with results obtained by other methods and with the conclusions of a study of Ca^{45} uptake in skull and teeth by Jarabak, Kamins, and Vehe.[13] In their investigation the autoradiographs were prepared from a single animal, and it is unfortunate that the age of the rat does not appear to have been recorded.

It has been of interest to observe the pattern of calcium uptake by the bone of the sockets around the maxillary molar teeth (Fig. 5). The heavier deposition of Ca^{45} on the lingual aspect of the teeth suggests that they migrate outward as the jaws increase in width, by periosteal accretion on the buccal aspect of the bone. This process is associated with a relative lack of Ca^{45} deposition on the internal aspect of the maxilla, which can be seen by histologic methods to be undergoing resorption changes. These modifications of jaw size are paralleled by growth at facial suture systems (Fig. 12).

The accumulation of Ca^{45} on the undersurface of the hard palate (Fig. 15), in complete contrast to the absence of deposition on the nasal aspect, indicates that the hard palate descends during the growing period. An increase in vertical dimension of the nasal cavities is one result, and the descent of the palate is accompanied by eruption of the teeth and appositional growth of maxillary alveolar bone.

Histologic controls prepared from littermates have been of assistance in the accurate interpretation of certain of the autoradiographs (Figs. 5–8). The serial contact method of autoradiography necessitates the progressive destruction of the labeled

tissue, so that it is not possible to prepare the same specimen by histologic means. In younger animals alternative autoradiographic methods have been attempted, in which both the histologic details and the sites of Ca^{45} uptake can be recorded from the same area. Because of difficulties involved in sectioning calcified bone, the results so far have been inferior to those herein described.

A preliminary investigation[15] which attempted to correlate the observations made by autoradiographic, alizarin, and histologic techniques shows that these methods are fully complementary. Many similarities were found in corresponding regions. Comparable findings were obtained in relation to the pattern of deposition and resorption, growth of the cartilaginous sutures of the base of the skull, and the fibrous sutures of the facial region.

SUMMARY

Thirty-five growing rats (birth to 62 days) were injected intraperitoneally with radioactive calcium (Ca^{45}) and killed at variable intervals after injection, ranging from 30 minutes to 77 days. Several pregnant rats were injected, to study the uptake of Ca^{45} by fetal tissues.

Serial contact autoradiographs, prepared from the dried skulls in various planes, show that accurate localization of the isotope occurs within 30 minutes of injection, permitting accurate identification of individual bony elements of the base of the skull and face. Likewise, radioactive material was taken up rapidly by fetal bones and dental tissues following placental transfer.

In the upper jaw, areas of heavy periosteal deposition of Ca^{45} on facial aspects are clearly defined from areas of relative inactivity on medial surfaces. The importance of the cartilages of the cranial base and nasal septum in increase in length of the skull and face is evidenced by the greater accumulation of Ca^{45} which occurs in these zones. Around the roots of the cheek teeth the pattern of Ca^{45} uptake suggests that these teeth migrate in an outward direction as the jaws increase in width.

It is concluded that Ca^{45} provides a useful addition to the methods available for studies of facial growth. Autoradiographic results are fully complementary with and substantiate those obtained by other techniques.

REFERENCES

1. Jarabak, J. R. Animal Cephalometry, *Angle Orthodont.*, **12**:87, 1953.
2. Selman, A. J., and Sarnat, B. G. A Headholder for Serial Roentgenography of the Rabbit Skull, *Anat. Rec.*, **115**:627, 1953.
3. Gaus, B. J., and Sarnat, B. G. Sutural Facial Growth of the *Macaca rhesus* Monkey: A Gross and Serial Roentgenographic Study by Means of Metallic Implants, *Am. J. Orthodont.*, **37**:827, 1951.
4. Brash, L. C. Some Problems in the Growth and Developmental Mechanics of Bone, *Edinburgh Med. J.*, **41**:305, 1934.
5. Moore, A. W. Head Growth of the Macaque Monkey as Revealed by Vital Staining, Embedding and Undecalcified Sectioning, *Am. J. Orthodont.*, **35**:654, 1949.
6. Craven, A. H. Growth in Width of the Head of the *Macaca rhesus* Monkey as Revealed by Vital Staining, *Am. J. Orthodont.*, **42**:341, 1956.
7. Levine, P. Certain Aspects of the Growth Pattern of the Rabbit's Skull as Revealed by Alizarin and Metallic Implants, *Angle Orthodont.*, **18**:27, 1948.
8. Erickson, L. C., and Ogilvie, A. L. Aspects of Growth in the Cranium, Mandible and Teeth of the Rabbit as Revealed through the Use of Alizarin and Metallic Implants, *Angle Orthodont.*, **28**:47, 1958.

9. LEBLOND, C. P., WILKINSON, G. W., BÉLANGER, L. F., and ROBICHON, J. Radio-autographic Visualization of Bone Formation in the Rat, *Am. J. Anat.*, **86**:289, 1950.
10. COMAR, C. L., LOTZ, W. E., and BOYD, G. A. Autoradiographic Studies of Calcium, Phosphorus and Strontium Distribution in the Bones of the Growing Pig, *Am. J. Anat.*, **90**:113, 1952.
11. TOMLIN, D. H., HENRY, K. M., and KON, S. K. Autoradiographic Study of Growth and Calcium Metabolism in the Long Bones of the Rat, *Brit. J. Nutrition*, **7**:233, 1953.
12. BOURNE, G. H. Autoradiography, *Biol. Rev.*, **27**:108, 1952.
13. JARABAK, J. R., KAMINS, M. M., and VEHE, K. L. Radioautographic Study of the Deposition of Calcium in the Cranium, Teeth and Mandible, *J.A.D.A.*, **47**:639, 1953.
14. DIXON, A. D. The Development of the Jaws, *D. Practitioner*, **9**:10, 1958.
15. DIXON, A. D., and HOYTE, D. A. N. Autoradiographic and Alizarin Techniques in the Study of Skull Growth, *J. Anat.* (*London*), **93**:589, 1959.

Experimental Studies on Neural Mechanisms of Bruxism

YOJIRO KAWAMURA, SHUSAKU TSUKAMOTO, and KIYOKATSU MIYOSHI

Department of Oral Physiology, Dental School, Osaka University
Joancho, Kitaku, Osaka, Japan

Bruxism has been treated as one of the important problems in the field of dental medicine. However, to our regret, it is only within the stage of an *antithetic* treatment, because the original mechanisms inducing the bruxism have not yet been well studied.

In this experiment a neural mechanism of gnashing (grinding of the teeth) was studied in rabbits as a preliminary attempt to solve the mechanisms of bruxism. The authors succeeded in inducing gnashing in rabbits by the electrical stimulation of the brain cortex. The high-frequency electrical stimulation on the strictly circumscribed cortical jaw motor area induced the characteristic gnashing. The characters of such a gnashing movement were analyzed.

Eleven male and female rabbits were used under light or deep anesthesia. The upper jaw and external auditory canals were firmly fixed. The site of application of the stimulus in the surface of the brain cortex was determined by exploring the chewing area and finding the point at which the gnashing response could be most readily elicited. For the stimulation, a platinum wire was used monopolarily, and square-wave pulses at frequencies of 60, 250, 750, and 1,000 c/s, pulse duration of 0.5 (or 1.0 msec.), were commonly employed. The duration of the periods of stimulation was about 10 seconds. Vertical and lateral movements of the jaw were simultaneously recorded separately on a smoked drum by means of levers suitably attached to a very small clamp applied to the lower incisors. In some experiments, noises of gnashing and activities of masseter and digrastric muscles were simultaneously recorded electrically by means of the ink-writing oscillograph, and noises of gnashing were recorded in a tape-recorder.

Gnashing was induced in rabbits by comparatively high-frequency stimulation from 60 c/s up to 1,000 c/s from the strictly circumscribed anteromedial aspects of the brain cortex. And this cortical region corresponded well with the cortical jaw motor area, excepting the anteromedial parts of it. This experimentally induced gnashing had a regular rhythm of 3–4 c/s independent of the stimulation frequency. This motion was slower than that of the natural mastication of rabbits or mastication-like jaw movements with rhythms of 5.6 c/s induced by low-frequency stimulation on this same cortical area. The gnashing motion consisted of the vertical and dominant lateral jaw movements, and the lateral deflection was more predominant than that of the usual masticatory movement. Gnashing was readily induced even by a low-frequency stimulation after topical application of strychnine nitrate.

Present results strongly indicate that an abnormal excitation of the cerebral cells in some parts of the cortical jaw motor area plays an important part in inducing gnashing.

Received for publication February 2, 1960; revised by authors June 30, 1960.

Comparison of Caries Incidence in Exercised and Immobilized Rats

RALPH R. STEINMAN, MORRIS BRUSSETT, and PETER TARTARYN
College of Medical Evangelists, School of Dentistry, Loma Linda, California

In a previous study (R. R. Steinman, M. G. Hardinge, and R. W. Woods, *J. D. Res.*, **37**:865, 1958), utilizing over 300 rats through three generations on eight different combinations of human foods, a direct correlation was found between the nutritional status of the rats and their incidence of caries. As the animals showed a deterioration from generation to generation, the teeth showed a corresponding deterioration. The purpose of the present study was to determine the possible effect of a physical deterioration brought about by other than nutritional means upon the incidence of incipient carious lesions in the albino rat.

Twenty-eight Osborn-Mendel rats from four litters were weaned at 21 days of age and placed on the following cariogenic diet: casein, 24; sucrose, 65; salts U.S.P. XIV, 4; corn oil, 5; dehydrated liver, 4; vitamin mix, 2; choline, 0.2 parts. These 28 animals were divided into two equal groups of littermates. One group of animals was placed in a large cage which allowed space to run. Food and water were given ad libitum. For 4 hours a day these rats were placed in a rotating cage which caused them to walk rapidly for 16 minutes and rest for 8 minutes. They had water available during this time. The other group of 14 rats was restricted by placing them within wire cages which allowed no freedom of movement, and food and water were available at all times.

All animals were sacrificed at 29 days of age and the jaws frozen immediately. The jaws were sectioned in the deep freeze by the method dveloped in this laboratory (R. R. Steinman, C. G. Hewes, and R. W. Woods, *J. D. Res.*, **38**:592, 1959). Incipient lesions were visualized in the dentin by using ninhydrin and X-ray, interproximal and occlusal.

The results concerning the effect of physical restraint upon the incidence of incipient carious lesions are shown in the table.

	No. of Animals	Died	No. Interprox.	No. X-Ray	Total Lesions	Av. Lesions per Animal
Immobilized....	14	1	39	18	192	14.0
Exercised.....	14	0	0	0	61	4.3

One of the immobilized animals died before the end of the experiment. The 13 remaining immobilized animals had a rather poor general appearance. They weighed 12 per cent less than those allowed to run free. The immobilization of the animals was obviously deleterious. The animals which were allowed to run and had exercise were superior in appearance to the confined animals.

The diets of these two groups of animals were identical. The feeding regimen was also the same for both groups of animals except during exercise. If the difference in weight is any indication, the exercised ones ate more than did the immobilized animals. The physical difference observed between the two groups of rats was apparently brought about by other than dietary means. Immobilization of rats is obviously a stress which could adversely affect the physical condition of the animals. The total lack of exercise could also profoundly effect the physical condition of the animals. It would appear logical that the observed difference between the two groups of rats was the result of stress and lack of normal exercise.

Earlier work (R. R. Steinman, M. G. Hardinge, and R. W. Woods, *J. D. Res.*, **37**:865, 1958) has shown that the resistance of rats to caries may be related to their physical status as affected by diet. The present study shows that the physical status may be altered by other than dietary means and that this in turn may have a profound effect upon resistance to decay. It would appear that the resistance of the host can be lowered by more than one method, which in turn may affect the incidence of caries.

This study was supported by Research Grant D-254 from N.I.H., U.S. Public Health Service.

Received for publication May 4, 1960; revised by authors July 1, 1960.

Positive Birefringence in Non-carious Enamel

BARBARA JEFFRIES WALLACE
Department of Biochemistry and Zollar Dental Clinic, University of Chicago
Chicago, Illinois

Von Ebner (*Arch. Mikroskop. Anat.*, **67**:18, 1905), Hals (*Fluorescence Microscopy of Developing and Adult Teeth* [Oslo: Norwegian Academic Press, 1953], p. 111), and Allan (*J. D. Res.*, **38**:1119, 1959) have described in mature and deciduous teeth islands of positive birefringence and immature matrix generally localized in the cervical enamel. In this laboratory non-carious areas of apparently hypomineralized enamel in the cervical region of many teeth have been found to bear a striking relationship to certain carious zones.

In a survey of sections cut serially from 93 permanent teeth from 59 patients in the Chicago area, this cervical effect was found in 80 per cent of the teeth. In no case were we able to correlate it with patient history of periodontal disease. The sections studied were immersed in glycerin, water, 95 per

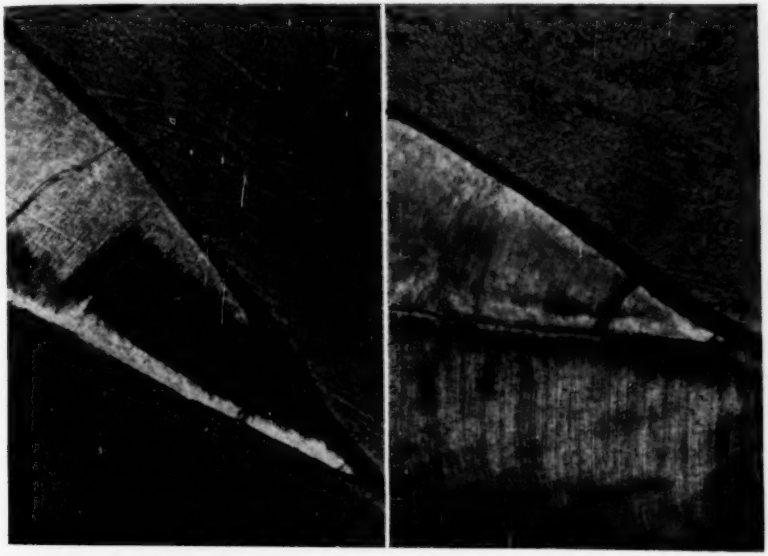


FIG. 1.—Cervical effect as seen in section immersed in glycerin. Polarized light. (Mag. ×67.)

FIG. 2.—Same section as shown in Fig. 1, immersed in distilled H_2O. Positive birefringence in cervical enamel has disappeared. Polarized light. (Mag. ×67.)

cent ethanol, and iodide solutions of R.I. 1.40–1.68, and fiewed in a polarizing microscope. Sections were also exposed to X-rays in a Phillips contact microradiographic unit.

In polarized light, immersed in glycerin or ethanol, the cervical effect is seen as a circumscribed spindle-shaped area of positive birefringence. The appearance with a Red I plate resembles considerably that of the so-called positive zone of many carious lesions. Immersion in water abolishes the form birefringence (Figs. 1 and 2). Imbibition with iodide solutions also abolishes all evidence of changed birefringence in these areas. The X-ray studies showed no decrease in radio-opacity in the region of the tooth corresponding to the area of cervical change.

These cervical areas are also similar to areas of anomalous birefringence in the occlusal enamel. Further studies are in progress to determine whether both regions are, in fact, hypomineralized and whether their anomalous behavior during imbibition and that of the carious-positive zone are physical consequences of the extent of mineralization or are the result of local chemical change.

Received for publication June 14, 1960.

This work was supported by Research Grant PHS-D-753 (C²) from the N.I.H., U.S.P.H.S.

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

Organized: New York City, December 10, 1920. Of those at the meeting of Organization, the following remain active members: Adolph Berger, Theodor Blum, A. H. Merritt, B. B. Palmer, L. M. Waugh.

Objects (*Const., Art. II*): The association has been established to promote broadly the advancement of research in all branches of dental science and in the related phases of the sciences that contribute directly to the development of oral health service, and as a whole; and, further, to encourage and facilitate co-operative effort and achievement by, and mutual helpfulness among, investigators in all nations in every division of stomatology to the end that dentistry may render cumulatively more perfect service to humanity.

Membership: Total (March, 1959), 1,003.

Eligibility.—Any person who conforms to the recognized standards of professional ethics and whose interest in dental science and dental research is shown by (*a*) research in process or conducted in the past, (*b*) scientific papers based upon original research presented before national or international meetings, or (*c*) scientific papers based upon original research and published in scientific journals is eligible for nomination to membership of the I.A.D.R.

Election.—New members are elected only on nomination by members and only by vote at the general meetings of the Association. Membership in a section or group does not confer membership in the Association. Nomination blanks for membership in this association may be obtained from the Secretary-Treasurer.

Endowment fund of the I.A.D.R. (as of October, 1959): $2,313.00.

OFFICERS OF THE ASSOCIATION

PRESIDENT Holmes T. Knighton, *Medical College of Virginia*
PRESIDENT-ELECT James A. English, *U.S. Navy*
VICE-PRESIDENT Seymour J. Kreshover, *National Institutes of Health*
SECRETARY-TREASURER Dan Y. Burrill, *Northwestern University*
EDITOR Frank J. Orland, *University of Chicago*

DIVISIONS AND SECTIONS

Division	*Section*	*President or Chairman*	*Secretary*	*Councillor*
North American	Ann Arbor	R. E. Moyers	K. Asgar	M. C. Crowley
	Baltimore	D. E. Shay	L. W. Gruber	E. C. Dobbs
	Birmingham	L. H. Schneyer	C. E. Klapper	S. B. Finn
	Boston	R. L. Glass	P. Goldhaber	H. H. Chauncey
	Chicago	J. R. Jarabak	A. A. Dahlberg	L. Fosdick
	Cleveland	S. W. Chase	M. M. Matt	P. E. Boyle
	Columbus	P. R. Weisenstein	S. Kolas	G. Green
	Houston	B. Levy	D. A. Hagerman	S. S. Arnim
	Indianapolis	R. W. Phillips	M. L. Swartz	J. C. Muhler
	Iowa City	W. Kern	D. E. Waite	A. K. Fisher
	Kansas City	B. K. Forscher	B. W. Warner	G. D. Roth
	Minnesota	A. Hampel	R. Stallard	L. Singer
	New York	H. J. Bartelstone	E. D. Goldsmith	G. Bevelander
	Pacific Northwest	B. S. Krause	B. J. Zeldow	L. Sreebny
	Philadelphia	H. Brenman	A. B. Hattler	N. B. Williams
	Pittsburgh	S. W. Leung	F. Draus	G. Cox
	Richmond	H. M. Syrop	G. W. Burke, Jr.	J. J. Salley
	Rochester, N.Y.	R. Lobene	H. A. Zander	E. Johansen
	St. Louis	J. W. Bynum	D. E. Flieder	J. R. Ring
	San Francisco		S. Epstein	W. C. Fleming
	Southern California	H. L. Dute	W. W. Wainwright	R. F. Sognnaes
	Toronto	D. L. Anderson	E. Harvold	G. Nikiforuk
	Washington, D.C.	R. M. Stephan	A. F. Forziati	W. C. Hess
	Wisconsin	C. W. Fairhurst	R. V. Winders	G. Ryge
British		M. A. Rushton	C. H. Tonge	A. I. Darling
Scandinavian	Denmark	P. O. Pedersen	J. J. Pindborg	J. J. Holst
	Norway	G. Toverud	I. Reichborn-Kjennerud	
	Helsinki	E. Tammisalo	K. Koski	
Japanese		S. Matsumiya	K. Enoki	G. Minoguchi
Not belonging to a Division	Johannesburg			A. J. Clement
	Mexico		F. R. Leycegui	
	Israel		S. Lewin-Epstein	
	Prague		F. Neumann	
	Austria, Vienna Center		E. Zitka	
	Australia	D. A. Cameron	G. S. Molyneux	A. R. Docking
Dental Materials Group		D. B. Mahler	M. L. Swartz	G. F. Paffenbarger

COUNCILLORS-AT-LARGE

Clifton O. Dummett — Ralph L. Ireland

*Supporting Associates, 1960–61**

OF THE INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH AND THE JOURNAL OF DENTAL RESEARCH

THE ACRALITE COMPANY, INC., New York, N.Y.
JULIUS ADERER, INC., New York, N.Y.
AMALGAMATED DENTAL COMPANY, LTD., London
ASTRA PHARMACEUTICAL PRODUCTS, INC., Worcester, Mass.
BLOCK DRUG COMPANY, INC., Jersey City, N.J.
L. D. CAULK COMPANY, Milford, Del.
COLGATE-PALMOLIVE COMPANY, Jersey City, N.J.
COLUMBIA DENTOFORM CORPORATION, New York, N.Y.
CRESCENT MANUFACTURING COMPANY, Chicago, Ill.
DENTISTS' SUPPLY COMPANY OF NEW YORK, York, Pa.
ENGELHARD INDUSTRIES, INC., Newark, N.J.
HANAU ENGINEERING COMPANY, INC., Buffalo, N.Y.
J. F. JELENKO & CO., INC., New York, N.Y.
H. D. JUSTI & SONS, INC., Philadelphia, Pa.
KERR MANUFACTURING COMPANY, Detroit, Mich.
ELI LILLY & CO., Indianapolis, Ind.
LORVIC CORPORATION, St. Louis, Mo.
MIDWEST DENTAL MFG. CO., Chicago, Ill.
THE J. M. NEY COMPANY, Hartford, Conn.
PRECIOUS METALS RESEARCH WORKS, INC., New York, N.Y.
THE PROCTER & GAMBLE COMPANY, Cincinnati, Ohio
RITTER COMPANY, INC., Rochester, N.Y.
ROCKY MOUNTAIN METAL PRODUCTS CO., Denver, Colo.
SCHERING CORPORATION, Bloomfield, N.Y.
SQUIBB INSTITUTE FOR MEDICAL RESEARCH, New Brunswick, N.J.
SURGIDENT, LTD., West Los Angeles, Calif.
S. S. WHITE DENTAL MFG. COMPANY, INC., Philadelphia, Pa.
THE UPJOHN CO., Kalamazoo, Mich.

ACADEMIC SUPPORTING ASSOCIATES

DENTAL MATERIALS GROUP, I.A.D.R.
Member—DENTAL MATERIALS GROUP, I.A.D.R.
CHICAGO SECTION, I.A.D.R.

* These were the Supporting Associates that made contributions in 1960 to the I.A.D.R. Treasury for support of the *Journal*. These contributions are in multiples of $100.